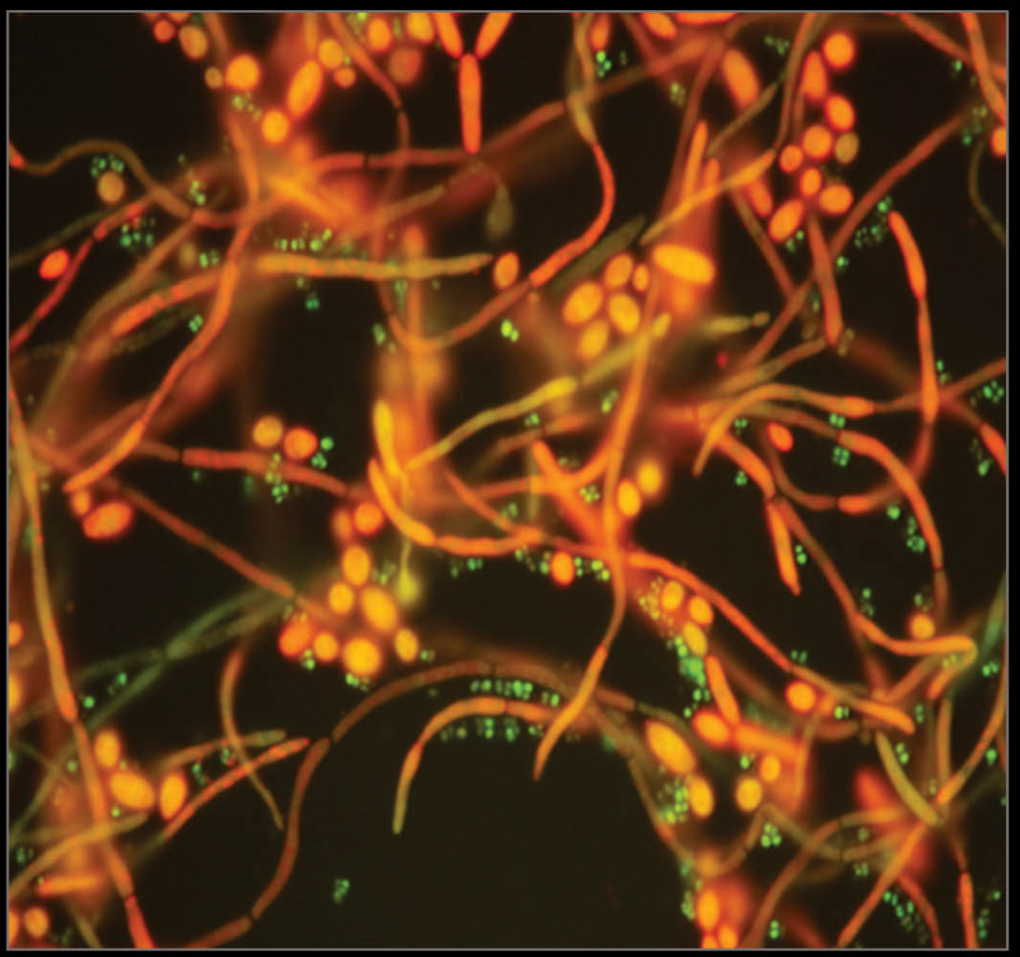
Candida and Candidiasis

SECOND EDITION



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Richard A. Calderone and Cornelius J. Clancy

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Cover: Candida albicans (red) and *Staphylococcus aureus* (green) biofilm stained with species-specific peptide nucleic acid (PNA)-FISH probes, demonstrating extensive adherence of *S. aureus* to the *C. albicans* hyphae. Courtesy Mary Ann Jabra-Rizk, University of Maryland, Baltimore.

Library of Congress Cataloging-in-Publication Data

Candida and candidiasis / edited by Richard A. Calderone, Cornelius J. Clancy. — 2nd ed.
p. ; cm.
Includes bibliographical references and index.
ISBN 978-1-55581-539-4
1. Candidiasis. 2. Candida. I. Calderone, Richard A., 1942- II. Clancy, Cornelius J.
[DNLM: 1. Candida. 2. Candidiasis. QW 180.5.D38]
QR201.C27C365 2012
616.9'693—dc23

2011025353

E-Book ISBN 978-1-55581-717-6
10.1128/ISBN978-1-55581-717-6

Printed in the United States of America
10 9 8 7 6 5 4 3 2 1
Address editorial correspondence to: ASM Press, 1752 N St., N.W., Washington, DC 20036-2904, USA.
Send orders to: ASM Press, P.O. Box 605, Herndon, VA 20172, USA.
Phone: 800-546-2416; 703-661-1593. Fax: 703-661-1501.
E-mail: books@asmusa.org
Online: http://estore.asm.org

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Preface

Over the past three decades, as one of the editors himself has witnessed, the experimental approaches and desired outcomes in the study of *Candida* spp. and the infections they cause naturally have changed. The overwhelming focus now is in molecular biology at a number of levels of research, such as genome comparisons and assessing virulence factors and host responses, as well as the promise of translational research into new antifungal drug discovery, diagnostics, and vaccines. The *Candida* community has been fortunate to witness the sharing of mutant libraries, strains, techniques, vectors, and probes; collaboration among laboratories seems to be increasing, a development that will be needed to solve the increasing complexity of research that requires interdisciplinary and "systems biology" approaches. Through genomics, we can now identify similarities and differences among *Candida* species, other human pathogenic and nonpathogenic fungi, and nonfungal species. "Omics" studies and databases are especially useful in designing new targets for drug discovery, but their application extends beyond this goal, to showing why pathogens are pathogens. That knowledge is in many cases at our fingertips.

This is the fourth in a series of volumes on *Candida* and candidiasis (candidosis) and the first that is coedited to reflect a more thorough treatise of human disease, treatment, and expectations in health care delivery. Each of the preceding books emphasized different things. *Candida and Candidosis* (University Park Press, Baltimore, MD, 1979) and *Candida and Candidosis: a Review and Bibliography*, 2nd Ed. (Baillière Tindall, Oxford, U.K., 1988), both written by Frank C. Odds, focused on the species that cause candidiasis, including their morphogenesis, virulence, and structure; the first of these books included special emphasis on the types of candidiasis. Dr. Odds gave us meaning and direction, a unification to address new problems that existed. The third book, Candida *and Candidiasis*, edited by Richard A. Calderone, was published in 2002 by ASM Press.

The present book, Candida *and Candidiasis*, 2nd Edition, is a natural extension of the previous three. In this volume are emphasized genomes and variability, host-pathogen interactions, antifungal resistance and new drug discovery, and evolving diagnostics. Variability among *Candida* species is described with regard to genomes, molecular adaptation to the external milieu whether in a host or in vitro, and sexuality of *Candida albicans*; we have learned how variability contributes to resistance to triazole drugs. Traditional areas of interest remain. For example, research in morphogenesis and the cell cycle (and, ultimately, growth) has provided new heights of understanding. Major advances in immune responses are also covered in this volume. Chapters discuss vaccine candidates in the community and how host responses may be useful in diagnosis of blood-borne candidiasis. Virulence attributes are now placed in the context of gene families. While the cell wall is critically included, it is represented more now as an entity that interacts with the innate host system. Broad representation of specific pieces of the cell is included, ultimately reflecting the current interests among like scientists. Biofilms, either mixed-species or monospecific, tell us much about the survival of the fungus in the host.

Discovery has continued, and translational research is moving toward attainable goals. But have we made a difference in increasing awareness of public health issues in candidiasis? An answer to that question is not easily discerned. Candidiasis is the third most frequent hospital-acquired infection. But who knows that fact, beyond the candidiasis community? In reality, new drug discovery features little more than remodeled old drugs. The search for that magic bullet that can kill all 100+ fungal pathogens still survives, at least partially, but this objective lacks sense and is not part of the paradigm in antibacterial drug discovery.

We must lose the notion that we cannot do better. The greatest risk for the next decade is that candidiasis research will become lost in the current economic times, at least in the United States. Emphasis on other important, nonfungal pathogens has overwhelmed the goal of controlling candidiasis, cryptococcosis, aspergillosis, the endemic mycoses, and dermatophytosis in public health. Solutions to this dilemma are not easy. To a much broader extent, we in this field must educate the public by choosing leaders among us, especially physician-scientists, who can testify to the importance of these diseases. These leaders should be called on to seize the interest of "think tanks" and other groups that influence policy makers. But also, each of us needs to remind our professional societies, the major advocates of microbiology, that this field demands equal attention with all the other pathogenic microorganisms, whether in newsletters, public education, or influence peddling.

Even within our discipline, we cannot keep up with everything. Both of us marveled at the outstanding research presented at the most recent "Candida and Candidiasis"

conference, held in Miami Beach, Florida, in March of 2010. That message should continue to be carried to the public, in a language that conveys the importance of these diseases. For this reason, just as the present volume offers the most current information in this critical field, new books on *Candida* and candidiasis should continue to present new discoveries and developments.

RICHARD A. CALDERONE
CORNELIUS J. CLANCY

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch1

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Candida: What Should Clinicians and Scientists Be Talking About?

BRAD SPELLBERG, KIEREN A. MARR, AND SCOTT G. FILLER

INTRODUCTION

The past several decades have witnessed a tremendous increase in our understanding of the virulence factors and host defense mechanisms operative during mucosal and systemic candidiasis. However, translation into clinically relevant interventions to prevent or treat candidal infections has lagged the impressive growth of our knowledge of disease mechanisms. While toxicity of antifungal therapy has improved with the advent of azoles and echinocandins, clinical cure and survival rates are not clearly better in patients with candidal infections treated with newer agents (17, 20, 29, 40, 62). Nor are we capable yet of predicting with a high degree of accuracy which patients will develop invasive candidiasis based on clinical criteria. As a result, we are not yet able to prevent these infections with any reliable, generally accepted strategy. Finally, diagnostics for candidal infections remain suboptimal, which further impedes our ability to treat infected patients. Advancing the state of the art of prophylactic and therapeutic interventions for mucosal and systemic candidal infections will require new perspectives on old problems.

"OPPORTUNISTIC" OR "ACCIDENTAL"? REDEFINING AN ESTABLISHED CONCEPT

Candida is often described as an "opportunistic" pathogen. However, the term opportunistic may be taken to imply that the predisposing risk factors for candidal infections involve defects in the host immune system. Furthermore, the term opportunistic connotes an intention on the part of the organism to cause disease when given the opportunity. Both of these implications may be misleading.

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While it is true that patients who develop candidal infections typically have specific predisposing risk factors, most of these patients do not have compromised immune systems, at least as detected by standard clinical testing. Specifically, dysfunction of the adaptive immune system is not of primary relevance to risk of disseminated candidiasis, since patients and mice with T- or B-lymphocyte deficiency are not at increased risk for systemic infection (2, 7, 12, 13, 23, 25, 58). In contrast, innate immune effectors (principally phagocytes) are critical to the host defense against systemic candidal infection, since neutropenia is a risk factor both for development of systemic candidiasis and also for worse outcomes from infection (11, 14, 19, 25, 28, 49). Nevertheless, only a minority (<20%) of patients who develop disseminated candidiasis are neutropenic (65). Furthermore, recent elegant experiments have demonstrated that neutropenia, by itself, is not sufficient to enable *Candida* to invade across the gut barrier and cause disseminated infection in mice (25, 63). Rather, even in the context of a neutropenic host, disruption of the normal anatomical barrier of intact gastrointestinal (GI) mucosa is necessary. Recognition of the requirement for gut disruption to enable onset of disseminated candidiasis was previously confounded by the fact that most chemotherapy agents that induce neutropenia also kill rapidly replicating GI epithelial cells. Only experiments using agents which selectively depleted neutrophils without affecting gut epithelium, disrupted gut epithelium without affecting neutrophils, or affected both were able to definitively untangle this pathogenetic web (25).

Furthermore, the most common clinical risk factors for disseminated candidal infections involve disruption of normal ecological and anatomical barriers which separate external compartments colonized by *Candida* from internal, normally sterile blood and tissue (49, 53). Such risk factors include the presence of central venous catheters, which provide direct access through the normally impenetrable skin barrier and into blood and also serve as a seedable endovascular nidus for persistent infection after fungemia resulting from translocation of fungus across the GI barrier; receipt of broad-spectrum antibacterials which facilitate fungal overgrowth; GI surgery which disrupts GI anatomical barriers;

and parenteral nutrition which results in gut epithelial barrier wasting and provides critical nutritional supplementation to facilitate fungal growth in the otherwise hostile blood environment. Hence, the primary opportunity for *Candida* to systemically invade a mammalian host involves the elimination of bacterial competitors, allowing fungal overgrowth, and breakdown of anatomical barriers at surfaces normally colonized by *Candida*, which results in fungal penetration to deeper tissues. It is also possible that subtle immune defects that reduce phagocyte antifungal activity may enable *Candida* to invade and proliferate in the host.

In contrast to systemic infection, oropharyngeal and esophageal candidiasis are typically associated with defects in cell-mediated, adaptive immunity (49). Neutropenia does not clearly predispose to mucosal infection. This discrepancy between the innate and adaptive immune requirements for the host defense against mucosal versus systemic candidiasis has never been adequately explained and merits investigation.

Although *Candida* seemingly coexists with the host in a contented, commensal state, on mucosal surfaces the organism is in a state of constant warfare with other microbial flora, and likely with the host. Any factor that weakens either the host or the competing microbial flora enables the fungus to overgrow and cause disease. For example, recently it has been found that patients who are heterozygous for a gene that specifies a nonfunctional version of dectin-1 have an increased rate of colonization with *Candida*. The protein dectin-1 plays a key role in the recognition of *C. albicans* by macrophages (54). These results suggest that the host immune system actively suppresses even commensal growth of the fungus. In turn, many, if not all, of the virulence factors of *Candida* likely evolved due to the selective pressure of the host immune system and the competing mucosal and skin microbiota.

While *Candida* has clearly coevolved with humans to persist on the mucosa and skin, disseminated candidiasis has only become common within the past 50 years, when advances in medical technology rendered patients susceptible to this disease. Therefore, it is virtually certain that virulence factors that enable the organism to cause invasive disease represent a subset of those that evolved to enable the organism to survive on the surface of the host. That is, when the organism invades or is mechanically propelled across weakened ecological or anatomical barriers, the defensive genetic programs that allow it to survive in the hostile environment of the mucosa and skin also enable it to proliferate in tissues of a susceptible host (8, 33, 56, 60, 79). Once inside the host, the organism begins digesting and absorbing nutrients from its environment (i.e., the host) to survive. A key variable that seems to transform a host colonized with *Candida* into a host with candidal disease is an alteration in one or more aspects of the host defense apparatus, including the normal bacterial flora, anatomical barriers, and immune system. Limited data suggest that it is also possible that a susceptible individual must be colonized with a strain of *Candida* that happens to have the correct collection of virulence factors to cause invasive disease (22), but this notion has not been investigated in depth.

Changing our perspective on this pathogen in terms of the events which transform colonization into disease, the organism's "intentions," and our normal defense mechanisms that check the fungus in its commensal environment may lead to important discoveries regarding how to prevent and treat these infections.

USE OF ANIMAL MODELS TO FACILITATE STUDY OF PATHOGENESIS DURING DISSEMINATED INFECTION

Two types of animal models have been used to study pathogenesis of disseminated candidiasis. The first is direct tail vein inoculation into the bloodstream of mice. The second involves various methods to persistently colonize the GI tracts of mice with *Candida* (mice are not normally colonized by the fungus), followed by compromising the animals to facilitate translocation of the fungus into blood across the gut barrier. These models clearly have relevance to human infection. Specifically, the direct tail vein infection model (i) recapitulates infection introduced into patients directly through catheters, (ii) has a clinical course similar to that of untreated disseminated candidiasis in humans, and (iii) has been predictive of efficacy of antifungal agents against systemic infection in humans (9, 30, 35, 48, 66). There are also established murine models of candidal enteral colonization resulting in GI tract translocation (10, 11, 25, 39). The latter models recapitulate the route that is likely to account for the majority of clinical infections (i.e., GI translocation) (11, 53). However, these models are more cumbersome to operate than tail vein infection.

Murine models have been very successful at identifying basic elements of candidal pathogenesis and host defense, as well as antifungal efficacy. Yet such models have limitations correlating with clinical infections. For example, fungal filamentation is a requirement for virulence in the murine (and fly) models, and non-*C. albicans* species which do not filament are hypovirulent relative to *C. albicans* (1, 15, 32, 68, 74). In contrast, morbidity and mortality of *C. glabrata* and *C. krusei*, which do not filament, are at least as high as or higher than those of *C. albicans* during human infection (50, 77). It is tempting to speculate that this increased mortality relative to *C. albicans* may reflect a sicker population of hosts who get *C. glabrata* and *C. krusei* infection in the clinical setting; it is difficult to quantify the attribution of infection towards mortality in a critically ill population with several host-driven risk factors for death. Thus, the murine models do not accurately recapitulate the mortality of infection caused by non-*C. albicans Candida* species, nor are they optimal for identifying virulence factors and host defense mechanisms operative against non-*C. albicans* species. In addition, there are significant differences between the murine and human immune systems.

Animal models available to date have been extremely helpful and have generated fundamental knowledge regarding pathogenesis and treatment of disseminated candidiasis. Additional insight into these processes may be gained by modifying existing murine models or developing alternative models that are more representative of human infection, particularly with non-*C. albicans* species. However, for development of optimal prophylactic or therapeutic strategies, it will be necessary to increasingly study the pathogenesis and immunology of *Candida* infections directly in humans.

BETTER DIAGNOSTICS ARE CRITICALLY NEEDED

For many decades, the only reliable diagnostic test for disseminated candidiasis has been blood cultures, which while highly specific are also highly insensitive and are known to be negative in up to half of cases (6, 24). Furthermore, blood cultures may take several days to become positive. It is known that optimal outcomes of disseminated candidiasis

occur when effective therapy is initiated within 12 to 24 h of the time the blood is drawn for culture (16, 41, 51). Cultures are rarely positive before this time frame. Hence, cultures are too insensitive and too slow to facilitate optimal medical care.

For the last several years, serum beta glucan has become available as a diagnostic test for disseminated candidiasis. The test has a moderate sensitivity (i.e., ~60 to 70%) and a high specificity (~80 to 90%) (18, 26, 44), resulting in a positive-likelihood ratio of ~5 and a negative-likelihood ratio of ~0.3. Unfortunately, clinical criteria can identify a population with no higher than a 5 to 10% pretest probability of developing disseminated candidiasis during follow-up (45, 46). Hence, assuming use of the maximally sensitive clinical criteria, resulting in a 10% pretest probability, a positive serum beta glucan test results in only a ~35% posttest probability of disseminated candidiasis, while a negative test results in a ~2% posttest probability of infection. The test is therefore far more useful if it is negative than if it is positive. Furthermore, even if a positive beta glucan test is accurate, and the patient indeed is candidemic, the beta glucan test does not identify the species of the fungus, which is required to optimize selection of antifungal therapy.

To optimize management of patients with disseminated candidiasis, new molecular diagnostic tests, whether antigen based, antibody based, or nucleic acid amplification based, are needed. Optimal tests not only would be highly sensitive and specific but also would be point-of-care tests or at least generate results within a few hours. Furthermore, such tests would be capable of distinguishing colonization from infection (since *Candida* is a commensal) and would identify the species of the fungus, and possibly even individual resistance genes. Clearly more research is needed to facilitate development of such tests.

OPTIMIZING CLINICAL OUTCOMES FROM DISSEMINATED CANDIDIASIS: TURNING TRADITION ON ITS HEAD

Superior Antifungal Therapy

The simplest conceptual means to improve morbidity and mortality from disseminated candidiasis is to create more effective antifungal therapies. While theoretically possible, the likelihood of finding nontoxic antifungal agents which are better at sterilizing fungi in blood and tissues than polyenes or echinocandins is unknown. Research is needed to identify new feasible targets for small-molecule development.

Another area of research which requires further investigation is the use of combination regimens to improve outcomes. One large-scale, prospective combination therapy study has provided critical insight into the potential for such strategies to improve outcomes (57). In that study, a combination of fluconazole plus amphotericin B deoxycholate resulted in a trend to superior survival versus fluconazole plus placebo in patients with candidemia. By preplanned subset analysis, the benefit of the combination therapy was entirely found in patients with an intermediate range of disease severity. This selective advantage for more aggressive therapy in moderately ill patients is likely because most patients with mild disease do very well on monotherapy, and most patients with severe disease have multi-organ failure and comorbidities, which are frequently not reversible and result in poor outcome even when appropriate antifungal therapy is administered. Hence, clinical studies of combination therapy may be best suited for investigation of patients with moderate severity of illness. New combinations, such as polyenes plus echinocandins, merit investigation to improve clinical outcomes based on synergy seen in preclinical models (5, 21, 42, 75). Furthermore, the potential for combination of immunotherapeutic strategies with antifungal strategies has been highlighted by the promising experience with the anti-heat shock protein antibody efungimab (47). Continued investigation of this and other potential combination strategies is critically needed.

Early Antifungal Therapy

Since the patient's severity of illness at baseline is predictive of mortality on therapy, and optimal outcomes are achieved when antifungal therapy is initiated within 12 to 24 h of drawing blood for culture (often before cultures are positive), it is logical to focus investigational strategies on those designed to facilitate early therapy. A recent prospective study failed to detect an advantage of early empirical therapy for suspected disseminated candidiasis (61). However, there was a trend to reduced culture-confirmed fungal infection in the empirically treated patients. Furthermore, the study had limited power to detect an impact of empirical therapy, given the expected low attack rate of disseminated candidiasis in the control arm. Finally, the composite primary efficacy endpoint was not clearly capable of distinguishing patients with disseminated candidiasis from those with myriad other causes of fever in the intensive care unit (ICU). Hence, this study cannot be considered conclusive, and further investigation of empirical therapy strategies for disseminated candidiasis is needed.

In designing an early therapy strategy, it is critical to recognize the limitations of currently available diagnostics. As has been emphasized previously (37), given a low pretest probability of disseminated candidiasis, resulting from our limited current understanding of how to predict who will get the infection, only an extremely specific test (almost certainly >95%) will be capable of identifying patients with a >75% posttest probability of disseminated candidiasis. Hence, until superior diagnostic tests become available, or mechanisms to accurately identify a population that has a very high prevalence of infection (>25%) become available, attempting to design investigations that positively select which patients should be enrolled in trials of early antifungal therapy will continue to be highly problematic.

While the tools available currently do not have adequate positive predictive value for disseminated candidiasis to positively select patients for enrollment in clinical trials, they do have highly useful negative predictive values (i.e., >98%). The high negative predictive values suggest potential utility of strategies that employ "de-escalation" antifungal therapy (37). In such a trial, patients would be enrolled initially based on clinical criteria suggesting risk for disseminated candidiasis (i.e., ~10% pretest probability). A blood test for serum beta glucan would be drawn at baseline, prior to randomization. The patients would then be randomized to receive antifungal therapy or placebo. Patients whose baseline, prerandomization beta glucans were negative would have their antifungal therapy stopped and be dropped from the primary efficacy analysis population. This concept of a de-escalation therapy strategy based on negative biomarkers for infection runs somewhat opposite to our traditional thinking for diagnostic-driven therapy in infectious disease clinical trials, but it may more accurately reflect the state of the art of available diagnostics and our limitations in knowing when to apply them.

Prophylactic Strategies

Need for Superior Predictors of Infection To Boost Attack Rates in Patients Enrolled in Trials

Given the considerable morbidity and mortality of disseminated candidiasis even with treatment, deployment of effective prophylactic strategies is highly desirable. However, enrolling sufficient numbers of patients to achieve an adequate power in a prophylactic study in which the attack rate of infection in the control arm is, at most, 10% will be challenging and very expensive. In contrast to development of new, early empirical therapy strategies, which will require improved diagnostic tools, deployment of a prophylactic strategy will require superior capability to identify patients who are at risk for disseminated candidiasis but do not yet have invasive disease. Given the thorough investigations already conducted to derive and validate clinical prediction rules (43), it is likely that further modification of those rules will result in only incremental, at best, advances over the predictors that are currently available. Hence, it is likely that incorporation of other forms of data into prediction rules will be necessary to improve their accuracy, and thus to support facile prophylaxis studies.

As mentioned, most (i.e., >80%) patients who develop disseminated candidiasis are not immunocompromised by standard clinical assays (49, 53). However, the very limited clinical data available suggest that poorly characterized, subtle defects in host defense may be present in patients who develop disseminated candidiasis. For example, one small retrospective study found that in postoperative patients, high levels of anti-*Candida* immunoglobulin E (IgE) were a specific diagnostic marker for invasive candidiasis, separating patients infected by, as opposed to colonized with, the organism (59). Since IgE is an in vivo biomarker of a Th2-mediated immune response (because class switching to IgE is dependent on interleukin 4), this retrospective study is concordant with murine data suggesting that type 2 immunity is antiprotective against systemic *Candida* infection (64). Furthermore, in another study, patients with *Candida* urinary infections who progressed to candidemia had elevated serum Th2 cytokine levels relative to patients whose infections remained localized in the bladder (70).

Simply administering *Candida* into the bloodstream does not necessarily result in established infection, because the reticuloendothelial system and neutrophils in normal mammalian hosts are so efficient at cleaning the blood of fungal burden. Within 30 min of intravenous inoculation of *Candida* in mice, rabbits, dogs, or humans, yeasts are retained within the reticuloendothelial system, especially in the liver (3, 34, 38, 69, 71). Indeed, during a single pass through the liver, a 1,000-fold reduction in fungal blood burden has been observed (69). Hosts whose cells have subtle defects in their ability to remove fungus from the blood, or to hunt down and kill fungus in tissues, are likely to be susceptible to the development of clinical infection at lower initial bloodstream inocula. If related to level of expression of cytokines, surface adhesins, or chemotactic factors or expression or function of pattern recognition receptors, such defects would go undetected by standard clinical immunology assays. Indeed, elegant clinical studies have confirmed the role of pattern recognition receptor polymorphisms in predisposing to mucosal candidiasis or candidal colonization (31, 54). Furthermore, Toll-like receptor 4 polymorphisms have been found at elevated rates in patients with candidemia versus control patients (76).

It is logical to assume that expression levels and polymorphisms of cytokines and pattern recognition receptors play a role in an individual patient's risk for developing established disseminated candidiasis. Incorporation of such factors into established clinical prediction rules will likely markedly enhance the predictive accuracy of such rules, resulting in a higher attack rate in patients enrolled in prophylactic and therapeutic clinical trials for invasive candidiasis. Hence, investigation of the immunology and immunogenetics of disseminated candidiasis in humans is critically needed.

Prophylactic Strategies

Four types of prophylactic strategies can be envisioned to prevent disseminated candidiasis. The first is a clinical systems-based approach to altering risk factors in individual patients. For example, Pronovost et al. have demonstrated that a checklist-based protocol for central venous catheter insertion and removal can decrease catheter-related bloodstream infections (55). While use of greater precautions during insertion of the catheter is highly unlikely to affect the risk of disseminated candidiasis, since the infection occurs after multiple days to weeks of hospitalization (78), earlier removal of catheters is of great promise in preventing these infections. Similarly, decreasing administration of total parenteral nutrition, greater antibiotic stewardship resulting in less inappropriate broad-spectrum antibacterial use, shorter hospital stays, improved prevention of renal failure, cancer prevention strategies, etc., are all likely to lead to a diminished risk of disseminated candidiasis. Investigations into such systems-based quality improvement strategies are greatly needed.

The second potential prophylactic strategy is the administration of antifungal agents in high-risk patients. Antifungal prophylaxis is highly effective and has become standard care to prevent candidal infections in patients who have received solid-organ or hematopoietic stem cell transplants, or in those with high risk, prolonged neutropenia. However, for nonneutropenic, nontransplant patients, the role of antifungal prophylaxis is not defined. While antifungal prophylaxis has been shown to be effective at reducing candidemia and mortality in one single center study of trauma/ICU patients (52), other studies have not been concordant, and the use of prophylactic antifungals to prevent *Candida* infections in the ICU setting remains controversial (49). Such prophylaxis may be reasonable on a case-by-case basis but in the long run will undoubtedly result in large shifts in fungal ecology, selecting out resistant species and, potentially, strains. Further investigation is needed to identify specific circumstances when the clinical benefits of antifungal prophylaxis outweigh the potential ecological harm.

The third potential prophylactic strategy is passive immunization or other immune-enhancing strategies. Passive immunization (i.e., administration of antibody to an at-risk host) may result in enhanced clearance of low-grade or initial fungemia, thereby increasing the necessary inoculum required to establish overt clinical infection. However, administration of antibody is typically more expensive than small-molecule or active vaccine strategies and is associated with a variety of potentially toxic effects, such as immune complex reactions (e.g., arthritis, nephritis, systemic inflammatory response syndrome, etc.), as well as anaphylactic reactions. Furthermore, the effect of passive immunization may still require functional phagocytes if the mechanism of protection is enhancement of opsonophagocytic killing of the fungus, as opposed to neutralization of virulence factors

or prevention of adhesion. Nevertheless, passive immunization is a proven strategy for the prevention of a variety of infections (mostly viral and toxin mediated) in exposed patients, and investigation into this type of strategy for candidal infections is warranted. Antibodies protective against disseminated candidiasis have been identified in preclinical models (72, 73) and merit clinical investigation. Similarly, nonspecific immune enhancing strategies, such as by administration of recombinant cytokines or agents that enhance innate immune function (e.g., Toll-like receptor agonists), are of great promise as prophylactic agents and should be investigated.

Finally, the fourth potential prophylactic strategy is active immunization. Active vaccines are typically relatively inexpensive and nontoxic. Disseminated candidiasis is a disease of prolonged hospitalization, with a mean time to onset of 3 weeks in hospital (78). Hence, there should be a window of opportunity to vaccinate most patients who are at risk for disseminated candidiasis, after identification of at-risk features and before onset of infection. In one study, mice which were not preimmune to *Candida* derived some protection from active vaccination as early as 1 week after the first dose of an effective vaccine (67). Since humans do have preexisting immunity to *Candida*, including to a promising protein vaccine candidate (4), the first dose of vaccination should result in a booster effect, with T-cell responses within 48 h. Thus, vaccination is of great promise to prevent these infections.

WHERE DO *CANDIDA* INFECTIONS BEGIN?

After many decades of study, we still lack a precise understanding of the frequency with which disseminated candidiasis begins from a skin versus a GI source (11, 53). It is clear that the GI tract can serve as a source for disseminated candidiasis, as was dramatically illustrated by the rapid onset of nearly fatal disseminated infection in a healthy volunteer who drank a *Candida* "milkshake" to prove that the fungus was nonpathogenic (27). In the limited investigations conducted to date, strains isolated from the blood of infected patients have been identical to strains harvested from the patients' GI tracts (36). Such data suggest, but do not prove, that in the large majority of clinical cases, the infection originates from a GI source rather than a skin source.

There have been virtually no investigations into the mechanisms by which *Candida* might cause disseminated infection from a skin source. For example, can the organism seed a catheter tip during initial placement of the catheter and subsequently cause infection? Does the organism get inoculated into the catheter during subsequent use because of inadequate hand washing, contaminated latex gloves, or contamination of the catheter port without alcohol cleansing prior to injection through the port? Investigations are needed on these and related issues of the origins of disseminated candidiasis.

There have also been only limited investigations into the primary source of strains that cause infections. For example, one study has suggested that even though the patients' GI tracts were the source for >90% of disseminated candidiasis cases studied, some strains may have been transmitted to patients' GI tracts from the hands of health care workers (27). The transmission dynamics, epidemiology, and ecology of *Candida* in the nosocomial setting are understudied. Such investigations are critical to enabling development of quality improvement and other clinical pathways that could interrupt the transmission cycle.

CONCLUSIONS

Much has been learned about how *Candida* causes infection and how the mammalian host defends itself from such infections. Much more will need to be learned if practical medical advancements are to be achieved based on the knowledge gained. Old paradigms about disease pathogenesis and methods of investigation should be challenged. Investigations into human biology, clinical epidemiology, immunology, and ecology are critically needed. New small-molecule and biological, prophylactic, and therapeutic strategies are needed. In order to appreciate potentially significant yet incrementally small clinical benefits of employing new strategies, better methods to perform clinical trials are needed. Finally, new clinical strategies, such as de-escalation therapy, infection control strategies, etc., are critically needed to improve our prevention and treatment of *Candida* infections.

REFERENCES

1. **Arendrup, M., T. Horn, and N. Frimodt-Moller.** 2002. In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. *Infection* **30:**286–291.
2. **Ashman, R. B., J. M. Papadimitriou, A. Fulurija, K. E. Drysdale, C. S. Farah, O. Naidoo, and T. Gotjamanos.** 2003. Role of complement C5 and T lymphocytes in pathogenesis of disseminated and mucosal candidiasis in susceptible DBA/2 mice. *Microb. Pathog.* **34:**103–113.
3. **Baine, W. B., M. G. Koenig, and J. S. Goodman.** 1974. Clearance of *Candida albicans* from the bloodstream of rabbits. *Infect. Immun.* **10:**1420–1425.
4. **Baquir, B., L. Lin, A. S. Ibrahim, Y. Fu, V. Avanesian, A. Tu, J. E. Edwards, and B. Spellberg.** 2010. Immunological reactivity of blood from healthy humans to the rAls3p-N vaccine protein. *J. Infect. Dis.* **201:**473–477.
5. **Barchiesi, F., E. Spreghini, A. W. Fothergill, D. Arzeni, G. Greganti, D. Giannini, M. G. Rinaldi, and G. Scalise.** 2005. Caspofungin in combination with amphotericin B against *Candida glabrata*. *Antimicrob. Agents Chemother.* **49:**2546–2549.
6. **Berenguer, J., M. Buck, F. Witebsky, F. Stock, P. A. Pizzo, and T. J. Walsh.** 1993. Lysis-centrifugation blood cultures in the detection of tissue-proven invasive candidiasis. Disseminated versus single-organ infection. *Diagn. Microbiol. Infect. Dis.* **17:**103–109.
7. **Black, C. A., F. M. Eyers, A. Russell, M. L. Dunkley, R. L. Clancy, and K. W. Beagley.** 1998. Acute neutropenia decreases inflammation associated with murine vaginal candidiasis but has no effect on the course of infection. *Infect. Immun.* **66:**1273–1275.
8. **Chiranand, W., I. McLeod, H. Zhou, J. J. Lynn, L. A. Vega, H. Myers, J. R. Yates III, M. C. Lorenz, and M. C. Gustin.** 2008. CTA4 transcription factor mediates induction of nitrosative stress response in *Candida albicans*. *Eukaryot. Cell* **7:**268–278.
9. **Clemons, K. V., M. Espiritu, R. Parmar, and D. A. Stevens.** 2006. Assessment of the paradoxical effect of caspofungin in therapy of candidiasis. *Antimicrob. Agents Chemother.* **50:**1293–1297.
10. **Clemons, K. V., G. M. Gonzalez, G. Singh, J. Imai, M. Espiritu, R. Parmar, and D. A. Stevens.** 2006. Development of an orogastrointestinal mucosal model of candidiasis with dissemination to visceral organs. *Antimicrob. Agents Chemother.* **50:**2650–2657.
11. **Cole, G. T., A. A. Halawa, and E. J. Anaissie.** 1996. The role of the gastrointestinal tract in hematogenous candidiasis: from the laboratory to the bedside. *Clin. Infect. Dis.* **22**(Suppl. 2)**:**S73–S88.

12. **Farah, C. S., S. Elahi, K. Drysdale, G. Pang, T. Gotjamanos, G. J. Seymour, R. L. Clancy, and R. B. Ashman.** 2002. Primary role for CD4+ T lymphocytes in recovery from oropharyngeal candidiasis. *Infect. Immun.* **70:**724–731.
13. **Fulurija, A., R. B. Ashman, and J. M. Papadimitriou.** 1997. Increased tissue resistance in the nude mouse against *Candida albicans* without altering strain-dependent differences in susceptibility. *J. Med. Vet. Mycol.* **35:**197–203.
14. **Fulurija, A., R. B. Ashman, and J. M. Papadimitriou.** 1996. Neutrophil depletion increases susceptibility to systemic and vaginal candidiasis in mice, and reveals differences between brain and kidney in mechanisms of host resistance. *Microbiology* **142**(Pt. 12)**:**3487–3496.
15. **Gale, C. A., C. M. Bendel, M. McClellan, M. Hauser, J. M. Becker, J. Berman, and M. K. Hostetter.** 1998. Linkage of adhesion, filamentous growth, and virulence in *Candida albicans* to a single gene, INT1. *Science* **279:**1355–1358.
16. **Garey, K. W., M. Rege, M. P. Pai, D. E. Mingo, K. J. Suda, R. S. Turpin, and D. T. Bearden.** 2006. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. *Clin. Infect. Dis.* **43:**25–31.
17. **Gomez, J., E. Garcia-Vazquez, C. Espinosa, J. Ruiz, M. Canteras, A. Hernandez-Torres, V. Banos, J. A. Herrero, and M. Valdes.** 2009. Nosocomial candidemia at a general hospital: prognosis factors and impact of early empiric treatment on outcome (2002–2005). *Med. Clin.* (Barcelona) **134:**1–5.
18. **Hachem, R. Y., D. P. Kontoyiannis, R. F. Chemaly, Y. Jiang, R. Reitzel, and I. Raad.** 2009. Utility of galactomannan enzyme immunoassay and (1,3) β-D-glucan in diagnosis of invasive fungal infections: low sensitivity for *Aspergillus fumigatus* infection in hematologic malignancy patients. *J. Clin. Microbiol.* **47:**129–133.
19. **Hamood, M., P. F. Bluche, C. De Vroey, F. Corazza, W. Bujan, and P. Fondu.** 1994. Effects of recombinant human granulocyte-colony stimulating factor on neutropenic mice infected with *Candida albicans*: acceleration of recovery from neutropenia and potentiation of anti-*C. albicans* resistance. *Mycoses* **37:**93–99.
20. **Horn, D. L., L. Ostrosky-Zeichner, M. I. Morris, A. J. Ullmann, C. Wu, D. N. Buell, L. L. Kovanda, and O. A. Cornely.** 2009. Factors related to survival and treatment success in invasive candidiasis or candidemia: a pooled analysis of two large, prospective, micafungin trials. *Eur. J. Clin. Microbiol. Infect. Dis.* **29:**223–229.
21. **Hossain, M. A., G. H. Reyes, L. A. Long, P. K. Mukherjee, and M. A. Ghannoum.** 2003. Efficacy of caspofungin combined with amphotericin B against azole-resistant *Candida albicans*. *J. Antimicrob. Chemother.* **51:**1427–1429.
22. **Ibrahim, A. S., F. Mirbod, S. G. Filler, Y. Banno, G. T. Cole, Y. Kitajima, J. E. Edwards, Jr., Y. Nozawa, and M. A. Ghannoum.** 1995. Evidence implicating phospholipase as a virulence factor of *Candida albicans*. *Infect. Immun.* **63:**1993–1998.
23. **Jensen, J., T. Warner, and E. Balish.** 1993. Resistance of SCID mice to *Candida albicans* administered intravenously or colonizing the gut: role of polymorphonuclear leukocytes and macrophages. *J. Infect. Dis.* **167:**912–919.
24. **Kami, M., U. Machida, K. Okuzumi, T. Matsumura, S. Mori Si, A. Hori, T. Kashima, Y. Kanda, Y. Takaue, H. Sakamaki, H. Hirai, A. Yoneyama, and Y. Mutou.** 2002. Effect of fluconazole prophylaxis on fungal blood cultures: an autopsy-based study involving 720 patients with haematological malignancy. *Br. J. Haematol.* **117:**40–46.
25. **Koh, A. Y., J. R. Kohler, K. T. Coggshall, N. Van Rooijen, and G. B. Pier.** 2008. Mucosal damage and neutropenia are required for *Candida albicans* dissemination. *PLoS Pathog.* **4:**e35.
26. **Koo, S., J. M. Bryar, J. H. Page, L. R. Baden, and F. M. Marty.** 2009. Diagnostic performance of the (1→3)-β-D-glucan assay for invasive fungal disease. *Clin. Infect. Dis.* **49:**1650–1659.
27. **Krause, W., H. Matheis, and K. Wulf.** 1969. Fungaemia and funguria after oral administration of *Candida albicans*. *Lancet* **i:**598–599.
28. **Lechner, A. J., K. E. Lamprech, L. H. Potthoff, T. L. Tredway, and G. M. Matuschak.** 1994. Recombinant GM-CSF reduces lung injury and mortality during neutropenic *Candida* sepsis. *Am. J. Physiol.* **266:**L561–L568.
29. **Leroy, O., J. P. Gangneux, P. Montravers, J. P. Mira, F. Gouin, J. P. Sollet, J. Carlet, J. Reynes, M. Rosenheim, B. Regnier, and O. Lortholary for the AmarCand Study Group.** 2009. Epidemiology, management, and risk factors for death of invasive *Candida* infections in critical care: a multicenter, prospective, observational study in France (2005–2006). *Crit. Care Med.* **37:**1612–1618.
30. **Leunk, R. D., and R. J. Moon.** 1979. Physiological and metabolic alterations accompanying systemic candidiasis in mice. *Infect. Immun.* **26:**1035–1041.
31. **Lev-Sagie, A., D. Prus, I. M. Linhares, Y. Lavy, W. J. Ledger, and S. S. Witkin.** 2009. Polymorphism in a gene coding for the inflammasome component NALP3 and recurrent vulvovaginal candidiasis in women with vulvar vestibulitis syndrome. *Am. J. Obstet. Gynecol.* **200:**303.e1–303.e6.
32. **Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink. 1997.** Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90:**939–949.
33. **Lorenz, M. C., J. A. Bender, and G. R. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot. Cell* **3:**1076–1087.
34. **Louria, D. B., N. Fallon, and H. G. Browne.** 1960. The influence of cortisone on experimental fungus infections in mice. *J. Clin. Investig.* **39:**1435–1449.
35. **MacCallum, D. M., and F. C. Odds.** 2005. Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. *Mycoses* **48:**151–161.
36. **Marco, F., S. R. Lockhart, M. A. Pfaller, C. Pujol, M. S. Rangel-Frausto, T. Wiblin, H. M. Blumberg, J. E. Edwards, W. Jarvis, L. Saiman, J. E. Patterson, M. G. Rinaldi, R. P. Wenzel, and D. R. Soll.** 1999. Elucidating the origins of nosocomial infections with *Candida albicans* by DNA fingerprinting with the complex probe Ca3. *J. Clin. Microbiol.* **37:**2817–2828.
37. **Marr, K. A., W. Leisenring, and E. Bow.** 2009. Empirical versus preemptive antifungal therapy for fever during neutropenia. *Clin. Infect. Dis.* **49:**1138–1139. (Author's reply, **48:**1139–1140.)
38. **Meister, H., B. Heymer, H. Schafer, and O. Haferkamp.** 1977. Role of *Candida albicans* in granulomatous tissue reactions. II. In vivo degradation of *C. albicans* in hepatic macrophages of mice. *J. Infect. Dis.* **135:**235–242.
39. **Mellado, E., M. Cuenca-Estrella, J. Regadera, M. Gonzalez, T. M. Diaz-Guerra, and J. L. Rodriguez-Tudela.** 2000. Sustained gastrointestinal colonization and systemic dissemination by *Candida albicans*, *Candida tropicalis* and *Candida parapsilosis* in adult mice. *Diagn. Microbiol. Infect. Dis.* **38:**21–28.
40. **Mora-Duarte, J., R. Betts, C. Rotstein, A. L. Colombo, L. Thompson-Moya, J. Smietana, R. Lupinacci, C. Sable, N. Kartsonis, and J. Perfect.** 2002. Comparison of caspofungin and amphotericin B for invasive candidiasis. *N. Engl. J. Med.* **347:**2020–2029.
41. **Morrell, M., V. J. Fraser, and M. H. Kollef.** 2005. Delaying the empiric treatment of *Candida* bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. *Antimicrob. Agents Chemother.* **49:**3640–3645.

42. **Olson, J. A., J. P. Adler-Moore, P. J. Smith, and R. T. Proffitt.** 2005. Treatment of *Candida glabrata* infection in immunosuppressed mice by using a combination of liposomal amphotericin B with caspofungin or micafungin. *Antimicrob. Agents Chemother.* **49:**4895–4902.
43. **Ostrosky-Zeichner, L.** 2009. Issues in the design and interpretation of antifungal drug trials in the critically ill. *Curr. Opin. Infect. Dis.* **22:**564–567.
44. **Ostrosky-Zeichner, L., B. D. Alexander, D. H. Kett, J. Vazquez, P. G. Pappas, F. Saeki, P. A. Ketchum, J. Wingard, R. Schiff, H. Tamura, M. A. Finkelman, and J. H. Rex.** 2005. Multicenter clinical evaluation of the (1→3) β-D-glucan assay as an aid to diagnosis of fungal infections in humans. *Clin. Infect. Dis.* **41:**654–659.
45. **Ostrosky-Zeichner, L., P. G. Pappas, S. Shoham, A. Reboli, M. A. Barron, C. Sims, C. Wood, and J. D. Sobel.** 2009. Improvement of a clinical prediction rule for clinical trials on prophylaxis for invasive candidiasis in the intensive care unit. *Mycoses* **54:**46–51.
46. **Ostrosky-Zeichner, L., C. Sable, J. Sobel, B. D. Alexander, G. Donowitz, V. Kan, C. A. Kauffman, D. Kett, R. A. Larsen, V. Morrison, M. Nucci, P. G. Pappas, M. E. Bradley, S. Major, L. Zimmer, D. Wallace, W. E. Dismukes, and J. H. Rex.** 2007. Multicenter retrospective development and validation of a clinical prediction rule for nosocomial invasive candidiasis in the intensive care setting. *Eur. J. Clin. Microbiol. Infect. Dis.* **26:**271–276.
47. **Pachl, J., P. Svoboda, F. Jacobs, K. Vandewoude, B. van der Hoven, P. Spronk, G. Masterson, M. Malbrain, M. Aoun, J. Garbino, J. Takala, L. Drgona, J. Burnie, and R. Matthews.** 2006. A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. *Clin. Infect. Dis.* **42:**1404–1413.
48. **Papadimitriou, J. M., and R. B. Ashman.** 1986. The pathogenesis of acute systemic candidiasis in a susceptible inbred mouse strain. *J. Pathol.* **150:**257–265.
49. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, and J. D. Sobel.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.
50. **Pappas, P. G., J. H. Rex, J. Lee, R. J. Hamill, R. A. Larsen, W. Powderly, C. A. Kauffman, N. Hyslop, J. E. Mangino, S. Chapman, H. W. Horowitz, J. E. Edwards, and W. E. Dismukes.** 2003. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin. Infect. Dis.* **37:**634–643.
51. **Patel, G. P., D. Simon, M. Scheetz, C. W. Crank, T. Lodise, and N. Patel.** 2009. The effect of time to antifungal therapy on mortality in candidemia associated septic shock. *Am. J. Ther.* **16:**508–511.
52. **Pelz, R. K., C. W. Hendrix, S. M. Swoboda, M. Diener-West, W. G. Merz, J. Hammond, and P. A. Lipsett.** 2001. Double-blind placebo-controlled trial of fluconazole to prevent candidal infections in critically ill surgical patients. *Ann. Surg.* **233:**542–548.
53. **Perlroth, J., B. Choi, and B. Spellberg.** 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med. Mycol.* **45:**321–346.
54. **Plantinga, T. S., W. J. van der Velden, B. Ferwerda, A. B. van Spriel, G. Adema, T. Feuth, J. P. Donnelly, G. D. Brown, B. J. Kullberg, N. M. Blijlevens, and M. G. Netea.** 2009. Early stop polymorphism in human DECTIN-1 is associated with increased *Candida* colonization in hematopoietic stem cell transplant recipients. *Clin. Infect. Dis.* **49:**724–732.
55. **Pronovost, P., D. Needham, S. Berenholtz, D. Sinopoli, H. Chu, S. Cosgrove, B. Sexton, R. Hyzy, R. Welsh, G. Roth, J. Bander, J. Kepros, and C. Goeschel.** 2006. An intervention to decrease catheter-related bloodstream infections in the ICU. *N. Engl. J. Med.* **355:**2725–2732.
56. **Ramirez, M. A., and M. C. Lorenz.** 2007. Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot. Cell* **6:**280–290.
57. **Rex, J. H., P. G. Pappas, A. W. Karchmer, J. Sobel, J. E. Edwards, S. Hadley, C. Brass, J. A. Vazquez, S. W. Chapman, H. W. Horowitz, M. Zervos, D. McKinsey, J. Lee, T. Babinchak, R. W. Bradsher, J. D. Cleary, D. M. Cohen, L. Danziger, M. Goldman, J. Goodman, E. Hilton, N. E. Hyslop, D. H. Kett, J. Lutz, R. H. Rubin, W. M. Scheld, M. Schuster, B. Simmons, D. K. Stein, R. G. Washburn, L. Mautner, T. C. Chu, H. Panzer, R. B. Rosenstein, and J. Booth.** 2003. A randomized and blinded multicenter trial of high-dose fluconazole plus placebo versus fluconazole plus amphotericin B as therapy for candidemia and its consequences in nonneutropenic subjects. *Clin. Infect. Dis.* **36:**1221–1228.
58. **Rich, R. R., T. A. Fleisher, W. T. Shearer, B. L. Kotzin, and H. W. J. Schroeder (ed.).** 2001. *Clinical Immunology: Principles and Practice*, 2nd ed. Mosby, New York, NY.
59. **Savolainen, J., A. Rantala, M. Nermes, L. Lehtonen, and M. Viander.** 1996. Enhanced IgE response to *Candida albicans* in postoperative invasive candidiasis. *Clin. Exp. Allergy* **26:**452–460.
60. **Schmidt, P., J. Walker, L. Selway, D. Stead, Z. Yin, B. Enjalbert, M. Weig, and A. J. Brown.** 2008. Proteomic analysis of the pH response in the fungal pathogen *Candida glabrata*. *Proteomics* **8:**534–544.
61. **Schuster, M. G., J. E. Edwards, Jr., J. D. Sobel, R. O. Darouiche, A. W. Karchmer, S. Hadley, G. Slotman, H. Panzer, P. Biswas, and J. H. Rex.** 2008. Empirical fluconazole versus placebo for intensive care unit patients: a randomized trial. *Ann. Intern. Med.* **149:**83–90.
62. **Sipsas, N. V., R. E. Lewis, J. Tarrand, R. Hachem, K. V. Rolston, I. I. Raad, and D. P. Kontoyiannis.** 2009. Candidemia in patients with hematologic malignancies in the era of new antifungal agents (2001–2007): stable incidence but changing epidemiology of a still frequently lethal infection. *Cancer* **115:**4745–4752.
63. **Spellberg, B.** 2008. Novel insights into disseminated candidiasis: pathogenesis research and clinical experience converge. *PLoS Pathog.* **4:**e38.
64. **Spellberg, B., and J. E. Edwards, Jr.** 2001. Type 1/type 2 immunity in infectious diseases. *Clin. Infect. Dis.* **32:**76–102.
65. **Spellberg, B., S. G. Filler, and J. E. Edwards, Jr.** 2006. Current treatment strategies for disseminated candidiasis. *Clin. Infect. Dis.* **42:**244–251.
66. **Spellberg, B., A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2005. Mice with disseminated candidiasis die of progressive sepsis. *J. Infect. Dis.* **192:**336–343.
67. **Spellberg, B., A. S. Ibrahim, L. Lin, V. Avanesian, Y. Fu, P. Lipke, H. Otoo, T. Ho, and J. E. Edwards, Jr.** 2008. An antibody titer threshold predicts anti-candidal vaccine efficacy even though the mechanism of protection is induction of cell-mediated immunity. *J. Infect. Dis.* **197:**957–971.
68. **Spellberg, B. J., D. Johnston, Q. T. Phan, J. E. Edwards, Jr., S. W. French, A. Ibrahim, and S. G. Filler.** 2003. Parenchymal organ, and not splenic, immunity correlates with host survival during disseminated candidiasis. *Infect. Immun.* **71:**5756–5764.

69. **Stone, H. H.** 1974. Studies in the pathogenesis, diagnosis, and treatment of *Candida* sepsis in children. *J. Pediatr. Surg.* **9:**127–133.
70. **Talluri, G., V. K. Marella, D. Shirazian, and G. J. Wise.** 1999. Immune response in patients with persistent candiduria and occult candidemia. *J. Urol.* **162:**1361–1364.
71. **Taschdjian, C. L., E. F. Toni, K. C. Hsu, M. S. Seelig, M. B. Cuesta, and P. J. Kozinn.** 1971. Immunofluorescence studies of candida in human reticuloendothelial phagocytes: implications for immunogenesis and pathogenesis of systemic candidiasis. *Am. J. Clin. Pathol.* **56:**50–58.
72. **Torosantucci, A., C. Bromuro, P. Chiani, F. De Bernardis, F. Berti, C. Galli, F. Norelli, C. Bellucci, L. Polonelli, P. Costantino, R. Rappuoli, and A. Cassone.** 2005. A novel glyco-conjugate vaccine against fungal pathogens. *J. Exp. Med.* **202:**597–606.
73. **Torosantucci, A., P. Chiani, C. Bromuro, F. De Bernardis, A. S. Palma, Y. Liu, G. Mignogna, B. Maras, M. Colone, A. Stringaro, S. Zamboni, T. Feizi, and A. Cassone.** 2009. Protection by anti-beta-glucan antibodies is associated with restricted beta-1,3 glucan binding specificity and inhibition of fungal growth and adherence. *PLoS One* **4:**e5392.
74. **Tsuchimori, N., L. L. Sharkey, W. A. Fonzi, S. W. French, J. E. Edwards, Jr., and S. G. Filler.** 2000. Reduced virulence of HWP1-deficient mutants of *Candida albicans* and their interactions with host cells. *Infect. Immun.* **68:**1997–2002.
75. **Tunger, O., H. Bayram, K. Degerli, G. Dinc, and B. C. Cetin.** 2008. Comparison of the efficacy of combination and monotherapy with caspofungin and liposomal amphotericin B against invasive candidiasis. *Saudi Med. J.* **29:**728–733.
76. **Van der Graaf, C. A., M. G. Netea, S. A. Morre, M. Den Heijer, P. E. Verweij, J. W. Van der Meer, and B. J. Kullberg.** 2006. Toll-like receptor 4 Asp299Gly/Thr399Ile polymorphisms are a risk factor for *Candida* bloodstream infection. *Eur. Cytokine Netw.* **17:**29–34.
77. **Viscoli, C., C. Girmenia, A. Marinus, L. Collette, P. Martino, B. Vandercam, C. Doyen, B. Lebeau, D. Spence, V. Krcmery, B. De Pauw, and F. Meunier.** 1999. Candidemia in cancer patients: a prospective, multicenter surveillance study by the Invasive Fungal Infection Group (IFIG) of the European Organization for Research and Treatment of Cancer (EORTC). *Clin. Infect. Dis.* **28:**1071–1079.
78. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317.
79. **Zakikhany, K., J. R. Naglik, A. Schmidt-Westhausen, G. Holland, M. Schaller, and B. Hube.** 2007. In vivo transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. *Cell. Microbiol.* **9:**2938–2954.

THE ORGANISMS, THEIR GENOMICS, AND VARIABILITY

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch2

2

An Introduction to the Medically Important *Candida* Species

GARY MORAN, DAVID COLEMAN, AND DEREK SULLIVAN

THE GENUS *CANDIDA*

Throughout the last quarter of the 20th century, *Candida* species assumed the mantle of the most common human fungal pathogen, a title they still hold as the 21st century progresses, despite challenges from emerging species. The genus *Candida* comprises of approximately 150 disparately related species that grow mainly as unicellular yeasts, with some species having the ability to grow in other forms, such as pseudohyphae and hyphae (e.g., *Candida albicans*). *Candida* spp. are found in a diverse range of environmental niches, with a relatively small number of species particularly associated with colonization and disease in humans (Table 1).

The history of *Candida* spp. and the infections they cause is long and has been reviewed extensively elsewhere (20, 100). Given the large size and the phylogenetic range of the genus *Candida*, it is perhaps not surprising that the taxonomy of *Candida* species is complex and a potential minefield for mycologists and clinicians. Historically, the taxonomy of fungi has relied on physical traits, particularly those associated with sexual reproduction. Therefore, since many *Candida* species have never been observed to have a sexual stage in their life cycle, these were originally assigned as Fungi Imperfecti (i.e., Deuteromycetes). However, since many species (including some human pathogens, e.g., *Candida krusei*) have been observed to reproduce sexually, there is potential for confusion concerning the nomenclature of species with an anamorphic (asexual) form and a teleomorphic (sexual) form. It has therefore been agreed that the name of the former can be used, hence *Candida lusitaniae* (the anamorph) rather than *Clavispora lusitaniae* (the teleomorph) (175). Other controversial topics that have been resolved include the merger of the genus *Candida* with the genus *Torulopsis* (183). In order for the name *Candida* to be used, it had to be designated a nomen conservandum; otherwise this book could have been entitled Torulopsis *and Torulopsiasis*.

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In recent years DNA sequence-based methods have helped to confirm taxonomic relationships within the genus and have been used to confirm that both sexual and nonsexual *Candida* species are ascomycetes (class Hemiascomycetes). These molecular methods are highly discriminatory and have revealed a high degree of genetic diversity within the genus. Molecular methods have shown that many of the medically important *Candida* species belong to a phylogenetic subgroup known as the CTG clade, a group of largely commensal yeast species that translate CTG as serine instead of leucine (50). This phylogenetic subgroup also contains the pathogenic species *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, and *C. guilliermondii*. However, other pathogenic *Candida* species, such as *C. krusei* and *C. glabrata*, have been shown to be distantly related to this group (Fig. 1). Molecular taxonomic methods are also directly responsible for the discovery of several novel *Candida* species of medical importance (e.g., *C. dubliniensis* [157], *C. metapsilosis*, and *C. orthopsilosis* [159]). While molecular taxonomic methods have undoubtedly proved very helpful in defining relationships among *Candida* species, their high discriminatory power should be used with caution, as they also have the potential to increase confusion due to difficulties associated with defining what delineates a microbial species. Ideally, phylogenetic differences in a putative novel species should be correlated with identifiable phenotypic traits.

THE CLINICAL IMPORTANCE OF *CANDIDA* SPECIES

The raison d'être for this book is the fact that *Candida* spp. continue to be significant human pathogens, particularly in patients with severe underlying disease and compromised immune defenses. But just how important is candidiasis amid the myriad of other microbial diseases that afflict humankind? *Candida* spp. are certainly very versatile organisms and have the ability to grow under a wide range of environmental conditions. This is reflected in the spectrum of diseases caused by *Candida*. The most common candidal infections occur on mucosal surfaces and include vulvovaginal and oropharyngeal candidiasis. In the case of vulvovaginitis,

TABLE 1 Medically relevant *Candida* species

Frequency	Organism
Common	*C. albicans*
	C. glabrata
	C. parapsilosis
	C. tropicalis
Infrequent	*C. krusei*
	C. dubliniensis
	C. guilliermondii
	C. lusitaniae
	C. rugosa
	C. orthopsilosis
	C. metapsilosis
Rare	*C. famata*
	C. inconspicua
	C. kefyr
	C. lipolytica
	C. norvegensis
	C. sake
	C. zeylanoides

Candida spp. are the most common cause of this infection in Europe and the second most common cause in the United States (67). Most women (approximately 75%) suffer from vulvovaginal candidiasis (VVC) at least once in life, with as many as 8% experiencing regular recurrent infections (139). VVC is primarily caused by *C. albicans* (approximately 90% of cases), while *C. glabrata* is the second most common cause (approximately 10%) of this infection (138). VVC is unusual among candidal infections in that infections can occur in immunocompetent individuals; however, risk factors are relatively ill-defined and include antibiotic use, contraception, pregnancy, and diabetes (Table 2).

Candida species are an important component of the normal flora of the human oral cavity, and if given the opportunity, these can overgrow and cause oropharyngeal candidiasis (OPC). Oral candidiasis is most often associated with underlying illness (e.g., diabetes) or immune dysfunction (particularly in human immunodeficiency virus [HIV]-infected individuals and AIDS patients); however, denture wearers, the elderly, and neonates are also susceptible to oral candidal infections (Table 2). OPC has been studied most intensively in HIV and AIDS patients, in whom it is the most commonly diagnosed opportunistic infection. There are several manifestations of OPC, including the pseudomembranous form, which is characterized by the presence of white curd-like lesions on oral mucosal surfaces and is better known as thrush. Other forms include erythematous candidiasis and angular cheilitis (32). The introduction of highly active antiretroviral therapy (HAART) in the mid-1990s has led to a marked decrease in the incidence of OPC in HIV-infected individuals (10, 25, 26). This is due to the reconstitution of host immune responses and also to inhibition of secreted aspartyl proteinase activity exhibited by *Candida* spp. (25, 55). OPC is most often caused by endogenous *Candida* species that are part of the normal oral flora. *Candida albicans* is

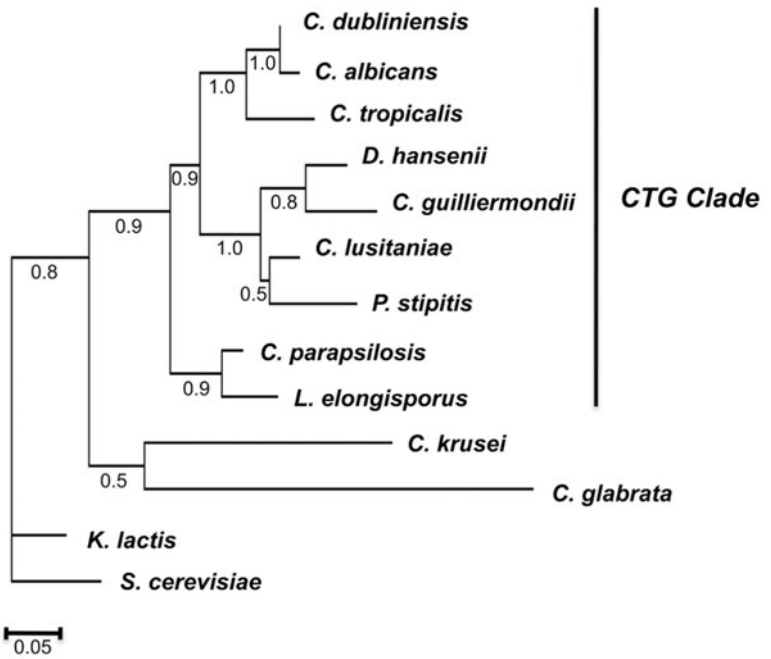

FIGURE 1 Phylogenetic tree showing the relationships between *Candida* yeasts of the CTG clade and other pathogenic *Candida* species. The tree is based on alignment of the amino acid sequences of the genes encoding enolase (Eno1p) from each species. Alignments were generated using MUSCLE using the default settings. Phylogenetic relationships were inferred with PHYML. Numbers at each node represent bootstrap values and indicate the number of times the arrangement shown occurred in 100 replicate trees. The bar under the tree represents a genetic distance of 5%. 10.1128/9781555817176.ch2f1

the most common cause of disease; however, other species (e.g., *C. glabrata*, *C. tropicalis*, and *C. dubliniensis*) are also associated with infection either alone or in combination with *C. albicans* (32, 156, 171). Since the reduction in the incidence in OPC among HIV-infected individuals in the developed world, there have been relatively few studies on its epidemiology. However, increased levels of *Candida* carriage and infection in the oral cavities of solid-organ transplant patients indicate that this cohort is also at particular risk of *Candida* infection (45).

While mucosal *Candida* infections are a significant clinical problem and cause considerable discomfort and reduced quality of life for infected individuals, it is when these organisms penetrate and traverse the epithelial barrier and enter the bloodstream that infections can become life threatening. These infections, collectively known as invasive candidiasis (IC), include candidemia and disseminated candidiasis, which can be acute or chronic in nature. *Candida* species are frequently reported as the fourth most common cause of bloodstream infection in the United States, behind staphylococci and enterococci (180); however, this is dependent on the patient population studied.

The gastrointestinal tract is home to a vast number of diverse microorganisms, including a variety of *Candida* species, which are believed to be the endogenous source of the majority of cases of IC (72, 109). Treatment with antibiotics can result in overgrowth of *Candida* leading to an increased bioburden, thus increasing the risk of IC. Other risk factors (Table 3) include chemotherapy, gastrointestinal surgery, and parenteral nutrition, which can result in damage to the integrity of the gut wall, thus facilitating the entry of *Candida* into the bloodstream. Exogenous *Candida* cells (for example, *C. parapsilosis* from the skin surface or the environment) can also be introduced directly into the vascular system via intravenous catheters. Another well-documented risk factor for IC is immunosuppression, in particular neutropenia (109). Once *Candida* cells are introduced into the bloodstream, they can be disseminated throughout the body to infect practically every organ, ultimately resulting in death if undiagnosed and untreated. Because IC occurs in patients who are already severely ill and have been hospitalized for protracted periods, it is difficult to accurately determine the rate of mortality attributed directly to IC. Recent estimates for IC-attributable mortality for adults range between 14.5% (184) and 49% (56), significantly higher than for other forms of bloodstream infections (e.g., those caused by *Staphylococcus aureus*) (56). Crude mortality rates associated with IC have remained in the region of 0.4 deaths per 100,000 over the period from 1997 to 2003 (112). It is often stated in the literature that the incidence of IC is continuing to increase. While this may have been true during the latter years of the 20th century, data from a major U.S. study suggest that the incidence of IC appears to have leveled off during the period from 1996 to 2003, remaining at a rate of approximately 23 infections per 100,000 population per year (112). In the same study, it was estimated that there are approximately 63,000 cases of IC in the United States each year, resulting in huge personal and financial costs, with the latter estimated at $1.7 billion per year in the United States in 2002 (178). Incidence rates for IC in Europe vary from country to country but tend to be lower than in the United States (75, 103). The reasons for the differences between the United States and Europe are unclear; however, differences in patient demographics and differences in the application of specific medical procedures may be partly responsible. Despite the high incidence rates reported for IC throughout the world, these are likely to be underestimations of the true extent of the problem due to the high rate of false-negative blood cultures associated with *Candida* infections. Clearly, IC continues to pose a significant clinical challenge despite the introduction of novel antifungal agents in recent years, and as the population of patients with risk factors continues to grow, it is likely that the incidence of IC will remain stubbornly high for the foreseeable future.

As in the case of other forms of candidiasis, the most common cause of IC is widely recognized as *C. albicans*, which is generally estimated to be responsible for approximately 50 to 60% of all IC cases (109, 112). Greater than 90% of cases of IC are caused by *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*, with the remaining dozen or so species only rarely associated with bloodstream infection (109, 112). The hierarchy of *Candida* species responsible for IC varies dramatically among countries and institutions and among patient groups. For instance, in North America and in Europe *C. glabrata* is most frequently identified as the second most common cause of IC, while in South America *C. parapsilosis* is often found to be the second most common species (69, 75, 103, 110, 161, 162). In recent studies from Asia the most commonly identified species associated with candidemia was *C. tropicalis* (27, 158). The reasons for the global differences in the distribution of the causes of IC are complex; however, differences in antifungal drug usage and cross-infection control and prevention practices are likely to be the most important factors. It is often reported that the incidence of *C. albicans* infections is decreasing relative to that of other *Candida* species and that non-*C. albicans Candida* species are emerging pathogens; however, it should be noted that in several studies the

TABLE 2 Major risk factors for mucosal *Candida* infections

Disease	Risk factors
VVC	Broad-spectrum antibacterial therapy
	Diabetes
	Corticosteroid therapy
	HIV infection
	Contraception use
	Hormone replacement therapy
OPC	HIV infection
	Broad-spectrum antibacterial therapy
	Immunosuppression
	Topical corticosteroid therapy (e.g., inhalers)
	Head and neck irradiation
	Denture wearing and poor denture hygiene
	Autoimmune polyendocrinopathy candidiasis ectodermal dystrophy syndrome (APECED)
	Uncontrolled diabetes mellitus
	Cancer chemotherapy
	Age of <1 yr
	Old age
	Trauma
	Nutritional deficiencies (e.g., iron)
	Xerostomia

TABLE 3 Major risk factors for IC

Colonization of the gastrointestinal tract with *Candida*
Broad-spectrum antibiotic therapy
Cancer chemotherapy
Gastrointestinal surgery
Parenteral nutrition
Central venous catheter
Neutropenia
Prolonged hospitalization
Burns
Renal failure
Organ transplantation

general distribution of species has not changed significantly in recent decades (e.g., see reference 179).

Candida albicans

Although all of the species in the CTG clade have been isolated from human subjects, *C. albicans* is, by far, the species most commonly recovered from human mucosal surfaces. Carriage rates for *C. albicans* of 30 to 60% are often reported for healthy human subjects, although carriage rate can increase with age and the presence of dental prostheses (31, 38). *Candida albicans* can be recovered from a broad range of anatomical locations, including the oral mucosa, the gut, the vaginal mucosa, and the skin (16, 31, 139). By using a variety of molecular typing techniques, numerous studies have confirmed that commensal and infecting isolates of *C. albicans* are genetically similar, supporting the notion that infecting strains generally originate from the patient's own flora (88, 101). Indeed, *C. albicans* is so well adapted to colonizing mucosal surfaces that a fine balance exists between the commensal state and overt infection. Although *C. albicans* is often classified as an opportunistic pathogen, infection is not strictly limited to individuals with compromised immunity, as *C. albicans* can cause superficial mucosal infections in otherwise healthy hosts due to disturbances in the normal flora as a result of antibiotic use or vaginal infection due to hormonal changes in otherwise healthy women.

Genetics and Mating

In terms of genetic tractability, *C. albicans* has traditionally been seen as a poor relation to *Saccharomyces cerevisiae*. The lack of a defined mating system and its diploid genome have for many years hampered classical and molecular genetic analysis. However, recent developments in genetic tools and genomics have led to an explosion in the number of studies investigating the genetics of this organism. Problems associated with diploidy have largely been overcome with the development of new selectable markers and more efficient methods of marker recycling (123). Within the last 10 years, the presumed asexual lifestyle of *C. albicans* has also been called into question by the discovery of a cryptic mating program in this organism. The discovery by Hull and Johnson that *C. albicans* contained a mating-type (*MAT*) locus similar to that in *S. cerevisiae*, including *MTL***a** and *MTL*α alleles on homologous chromosomes, led to later studies that demonstrated that strains homozygous at the mating locus could mate in vitro and following inoculation in mice (62, 63, 91). Subsequently, naturally occurring, **a**/α heterozygous strains that undergo homozygosis at the mating locus have been shown to mate, and that mating competency is associated with a switch to an "opaque" cell type in homozygous strains (182). Although genomic comparisons have shown that *C. albicans* contains many of the genes required for meiosis, no meiotic program has been identified thus far, and tetraploid mating progeny can only regain diploid status through a process of random chromosome loss (51). Despite intense investigation of this process over the last 10 years, firm evidence that mating occurs in natural populations of *C. albicans* remains elusive. Genetic analysis of *C. albicans* population structures confirms that reproduction in this species is largely asexual, with at most a minor input from sexual recombination (17).

Pathogenicity of *Candida albicans*

Traditionally, the high prevalence of *C. albicans* in the human population relative to other *Candida* spp. is attributed to a range of characteristics that are absent or partly absent in other yeast species. Dimorphism, the capacity to interconvert between yeast and hyphal morphologies, is often cited as being crucial for the success of *C. albicans* (Fig. 2). Hyphal forms have been associated with greater adherence, and in some instances tissue invasion, although yeast forms have also been observed in tissues in vivo (48, 122). Firm evidence that the yeast-to-hypha transition is crucial for pathogenicity was first presented in 1997 by Lo et al., who demonstrated that a nonfilamentous mutant strain (in which the transcription factor-encoding *EFG1* and *CPH1* genes were disrupted) was avirulent in a mouse model of infection (85). In 2003, Saville et al. showed, using strains in which morphology can be controlled with doxycycline, that yeast cells have the capacity to disseminate during murine hematogenous infections, but tissue invasion and lethality are reduced when filamentation is prevented. These data support the notion that growth in the yeast form plays a role in spread and dissemination of infection, but hyphal forms are necessary for widespread tissue invasion (132). However, subsequent studies with the same strains showed that yeast cells could also display a pathogenic effect leading to lethality in severely immunosuppressed mice (85). Another alternative growth form of *C. albicans*, the chlamydospore (Fig. 2), is formed when the organism is grown in vitro on certain nutrient-poor media (3). *Candida dubliniensis* is the only other member of the CTG clade that readily forms these structures (30, 147). However, no conclusive evidence for the occurrence of these structures during infection of human tissues has ever been presented. Recent evidence suggests that these structures may be a dormant growth form of the fungus formed under nutrient-depleted conditions that may be reactivated upon stimulation with serum or nutrients (30).

Both yeast and hyphal morphologies of *C. albicans* have been reported to exhibit greater adherence than non-*C. albicans Candida* species, with the exception of *C. dubliniensis*, which some reports claim is at least as adherent as *C. albicans* (40, 70). Extracellular hydrolytic enzymes such as proteinases and phospholipases may play diverse roles in tissue destruction, nutrient acquisition, adherence, and protection from host defenses (48, 98). The ability to tolerate a wide range of stressful environmental conditions is often cited as contributing to virulence, particularly the ability to tolerate wide ranges of pH, oxidative, and osmotic stresses. In the past, the ability of *C. albicans* to adapt to such a wide variety of niches has been associated with the property of phenotypic switching (137). Switching occurs in *C. albicans* and

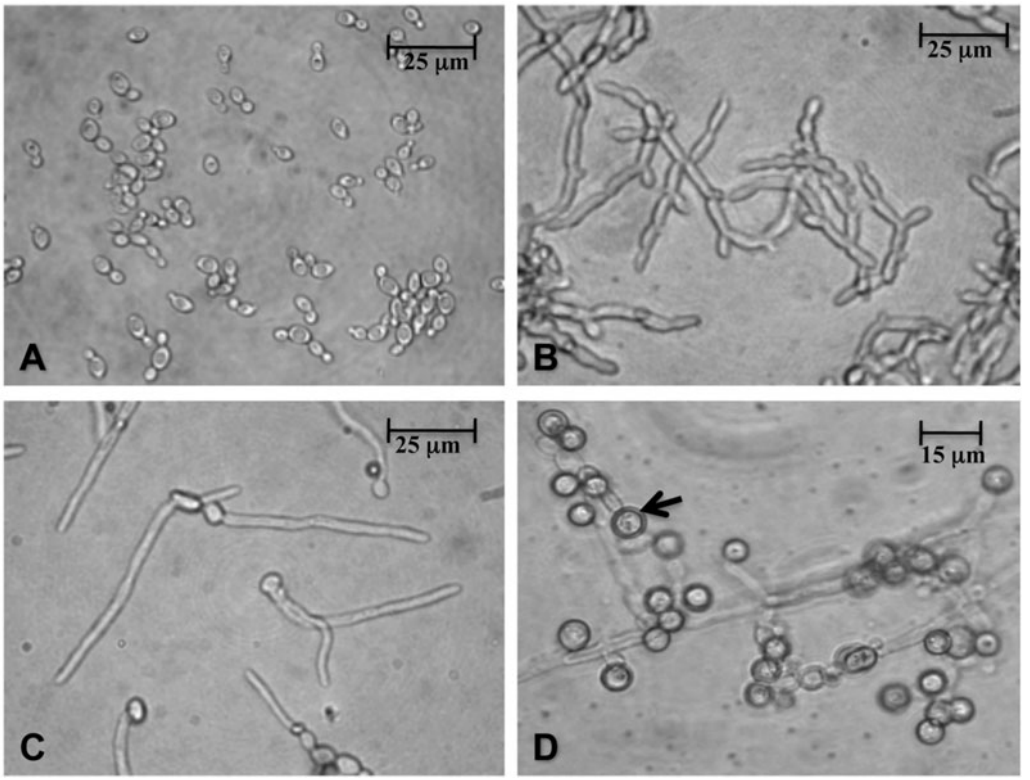

FIGURE 2 Photomicrographs showing the different growth morphologies of C. *albicans* yeast cells (A), pseudohyphae (B), hyphae (C), and chlamydospores (D; one example is indicated by an arrow). 10.1128/9781555817176.ch2f2

C. *dubliniensis* and manifests as high-frequency, reversible changes in colonial morphology. A repertoire of various colonial morphologies has been described for C. *albicans*; however, a firm molecular basis for generalized switching has yet to be established, although it is likely to involve regulators of cellular morphology which affect colonial appearance. A more specific switching system, the white-opaque switch, has been the subject of intense research due to its role in the mating process (140).

Molecular Analysis of Candidal Virulence

It is likely that the characteristics described above are adaptations that have evolved to promote colonization of mucosal surfaces and may explain why C. *albicans* is a more significant part of the oral flora than non-C. *albicans Candida* species. However, some debate exists as to whether characteristics such as these can be described as true virulence factors (102). Over the last 20 years, C. *albicans* has undergone intense investigation using the tools of genetics and molecular biology to understand the molecular basis of pathogenesis. The *Candida* Genome Database now contains over 200 genes annotated with the gene ontology term pathogenesis. However, many of these are involved in metabolism, cell structure, or regulation of morphology. Few, if any, virulence factors have been identified which satisfy molecular Koch's postulates, as orthologues of these genes are often present in nonpathogenic yeasts. In addition, recent comparative genomic investigations have shown that many virulence-associated genes, such as the agglutinin-like sequence (*ALS*) genes and the secreted aspartyl proteinase (*SAP*) genes, are found across the CTG clade, although the repertoire of these genes appears to be far greater in C. *albicans* (19, 64). Problems with defining virulence factors in C. *albicans* have been confounded by the use of auxotrophic strains to construct homozygous mutant derivatives. Problems associated with the use of the *URA3* gene in genetic selections have been well documented, and altered expression levels of Ura3 have a marked effect on growth and filamentation (146). In fact, recent investigations have seen *Candida* investigators undertake a hard reappraisal of much of the earlier molecular genetic work on virulence genes in this organism. This is best exemplified by studies with the adhesin-encoding gene *HWP1* (145). Initial studies by Tsuchimori et al. in 2000 indicated that a homozygous deletion of the *HWP1* gene attenuated virulence (163). However, subsequent studies have indicated that the reduced virulence of these strains was largely due to inefficient expression of the *URA3* marker gene (134). Other candidates for the mantle of virulence factor in C. *albicans* include the Saps, which have been implicated in both systemic and superficial infections (98). However, recent studies have indicated that these genes may not be required for invasion of a reconstituted human oral epithelium in vitro (77). Further studies will be required to determine whether this is due to a deficiency in the in vitro model system, the host strain (C. *albicans* SC5314, which is a poor colonizer of mucosal tissues in vivo), or a true reflection of the limited role of Saps in epithelial colonization. One molecule that has been the subject of intense investigation in recent years is Als3, a hypha-specific member of the Als protein family (59, 119). Several studies have implicated Als3 as an invasin required for penetration of host tissues by an endocytic mechanism, similar to bacterial

molecules like InlA of *Listeria monocytogenes* (119). Additional evidence has been presented suggesting a role for Als3 in the acquisition of iron from host ferritin (4). Furthermore, Als3 is unique to *C. albicans* among the CTG clade of organisms (64), and vaccination with a recombinant form of the molecule protects mice from lethal hematogenous infection (143). These findings suggest that Als3 may be unique among *C. albicans* virulence attributes in that it satisfies molecular Koch's postulates and may be classified as a bona fide virulence factor.

Although the search for virulence attributes in *C. albicans* is a valid one, and may lead to the discovery of potential vaccine targets such as Als3, many of the models used to study these attributes ignore the basic fact that *C. albicans* is a commensal of mucosal surfaces. Models of infection generally do not investigate mucosal colonization, and some, such as the systemic mouse model, actively bypass this stage of infection. Recently, newer models of oral colonization have been developed and some older models of gastrointestinal infection have been reappraised, which hopefully in the future can be used to broaden our understanding of candidal pathogenesis to include mucosal colonization and penetration (121, 150).

Antifungal Drug Susceptibility

In general, the majority of *C. albicans* isolates are fully susceptible to all major classes of antifungal agents, including the azoles, echinocandins, and polyenes. The exception to this is flucytosine, which is no longer used as monotherapy for *C. albicans* infection due to the high levels of intrinsic resistance (43). Prior to the advent of HIV and AIDS, resistance of *C. albicans* to the commonly used azole antifungals was rarely reported. However, the AIDS pandemic demonstrated that colonizing *C. albicans* strains in patients undergoing long-term azole therapy can acquire resistance by a number of mechanisms. Parallel studies by Dominique Sanglard and Theodore White demonstrated the role of the multidrug transporters *CDR1* and *MDR1* and point mutations in the *ERG11* gene, encoding the cytochrome P-450 lanosterol 14α-demethylase, as being important in the development of fluconazole resistance in *C. albicans* isolates from HIV-infected patients. Fortunately, most isolates of *C. albicans* recovered from bloodstream infection remain highly susceptible to fluconazole and voriconazole (110). Although resistance to caspofungin has been documented, this remains rare in *Candida albicans* (106).

Candida glabrata

Candid glabrata, formerly known as *Torulopsis glabrata*, grows as oval yeast cells and, unlike many other medically important *Candida* species, does not exhibit dimorphism. It is a haploid yeast and is phylogenetically more closely related to *Saccharomyces cerevisiae* than to other clinically important *Candida* species (74). An inspection of the *C. glabrata* genome identified several genes that are involved with mating and meiosis, and so this fungus may possibly have a cryptic sexual stage (18, 144, 181). *Candida glabrata* has been increasingly implicated in human infection over the last two decades following the advent of HIV infection and AIDS and the widespread use of immunosuppressive therapies (27, 32, 35, 41, 49, 57, 76, 78, 82, 103, 110, 116, 149, 179). It can be found as part of the normal commensal flora and is frequently isolated from the oral cavity in combination with other *Candida* species, most commonly *C. albicans* (82). *Candida glabrata* exhibits low virulence in animal models of infection (9). Colonization of the oral cavity by *C. glabrata* increases with age, and *C. glabrata* is the second most frequently isolated oral species after *C. albicans* in diabetics (65, 86). A number of studies of patients with advanced cancer reported that *C. glabrata* was the most prevalent oral non-*C. albicans Candida* species identified (12, 82). Oral colonization by *C. glabrata* is also very common in HIV-infected individuals. *C. glabrata* has been identified as the predominant yeast recovered from dentures in elderly patients with chronic atrophic candidiasis, very likely because of the enhanced ability of this species to adhere to acrylic surfaces relative to *C. albicans* (86). A study by Luo and Samaranayake demonstrated that cell surface hydrophobicity promotes the adherence of *C. glabrata* to denture acrylic surfaces and that this organism exhibits a fourfold-higher cell surface hydrophobicity and a twofold-greater tendency to adhere to denture acrylic than does *C. albicans* (90). Adhesion of *C. glabrata* to host cells appears to involve a family of lectins encoded by the *EPA* (epithelial adhesin) genes. This gene family is contained in subtelomeric loci, where they are subject to transcriptional silencing. The normally silent *EPA* genes are expressed during murine urinary tract infection, and expression in vivo appears to be due to nicotinic acid limitation, a precursor of NAD^+. *Candida glabrata* is a nicotinic acid auxotroph, and it appears that the organism uses this metabolic deficiency as a mechanism of sensing the host environment through a reduction in NAD^+ availability (44).

Candida glabrata has emerged as a significant cause of systemic fungal infection. Surveillance studies from the United States reported that the rate of *C. glabrata* bloodstream infection increased from 14 to 18% between 1992 and 2001 (110). Other studies reported that *C. glabrata* was the second most common *Candida* species after *C. albicans* responsible for disseminated infection in the United States between 1997 and 2001, accounting for up to 24% of bloodstream isolates (41). *Candida glabrata* has been reported to be the most common (20.1%) non-*C. albicans Candida* species responsible for bloodstream isolates in Canada between 1992 and 2001; however, it was less common in Europe (12.9%) and Latin America (7.5%) during the same period (110). A study from the United States by Hachem et al. reported that the rate of *C. glabrata* infection increased to 31% in patients with hematologic malignancies between 1993 and 2003, compared to 12% in a previous study at the same center between 1988 and 1992 (57). In contrast, a study from the ARTEMIS Surveillance Program, a global antifungal surveillance network, reported only small increases in the rate of *C. glabrata* infection, from 11 to 12% between 1997 and 2003 (116).

A number of studies that reported an increase in the rate of *C. glabrata* infection associated this phenomenon with widespread fluconazole prophylaxis in chronically immunosuppressed populations (76). However, the increased prevalence of *C. glabrata* in the elderly, who do not routinely receive antifungal prophylaxis, is more likely due to a reduction in the efficacy of host immunity due to aging. *Candida glabrata* is known to exhibit intrinsic low-level fluconazole resistance and to readily acquire or develop high-level resistance with cross-resistance to other azole drugs following exposure to fluconazole (68, 76, 149, 167). Several molecular mechanisms that contribute to fluconazole resistance in *C. glabrata* clinical isolates have been identified, including overexpression of *CgERG11* (encoding the cytochrome P-450 lanosterol 14α-demethylase); upregulation of the ABC transporter-encoding genes *CgCDR1*, *CgCDR2*, and *CgSNQ2*; and loss of mitochondrial function by the induc-

tion of respiration-deficient mutants (13, 28, 58, 66, 97, 130, 164). Clinical isolates of *C. glabrata* exhibiting reduced susceptibility or resistance to caspofungin have also been reported (28, 71). Substitutions in the *CgFKS1* and *CgFKS2* genes, encoding catalytic subunits of the 1,3-β-D-glucan synthase, were associated with a caspofungin reduced-susceptibility or resistance phenotype (53). Resistance to polyenes in *C. glabrata* clinical isolates has also been reported (148, 169, 170). The rapid development of antifungal drug resistance in *C. glabrata* is facilitated by the haploid nature of its genome. This poses very real clinical problems and reduces therapeutic options for the effective treatment of this emerging opportunistic pathogen, especially in immunosuppressed populations.

Candida parapsilosis

Candida parapsilosis grows as round, oval, or elongated yeast cells and as pseudohyphae. It was originally thought to be the anamorph of the ascomycete yeast *Lodderomyces elongisporus*, although studies have clearly shown that these yeasts represent distinct taxa (87, 99, 128). Unlike *C. albicans* and *C. dubliniensis*, *C. parapsilosis* cannot form true hyphae. It is a naturally diploid organism (19). Isolates of this yeast are frequently recovered from skin and nails, including the hands of health care personnel, where it forms part of the normal human commensal flora (80, 162, 166). It is also frequently recovered from the surfaces of plastic medical devices such as intravascular catheters and parenteral nutrition lines as well as other prosthetic devices (162, 166). However, *Candida parapsilosis* can also be isolated from a wide variety of environmental sources, including soil, freshwater and marine water, and plants, as well as from insects (23, 24, 52, 94, 151, 162, 166). *Candida parapsilosis* is considered to be an emerging yeast pathogen and is increasingly associated with a wide range of infections (reviewed by Trofa et al. [162]). *Candida parapsilosis* is frequently isolated from physical surfaces in the hospital environment, making it unique among *Candida* species (166). In this regard it is particularly associated with nosocomial infections, especially among premature neonates, patients in intensive care units, and patients receiving parenteral nutrition (21, 22, 37, 60, 61, 80, 81, 92, 166). The predominant risk factors for *C. parapsilosis* bloodstream infection are associated with iatrogenic and/or nosocomial factors, and the majority of *C. parapsilosis* infections are due to exogenous acquisition (142, 166). Since 1990 there has been an increased incidence of *C. parapsilosis* infection in many parts of the world, and it is the second or third most common yeast species recovered from bloodstream infection in Asia and Latin American countries (129). Bloodstream infection caused by *C. parapsilosis* is also now common in Europe and in the United States (reviewed by van Asbeck et al. [166]).

Candida parapsilosis exhibits lower virulence than *C. albicans*, most likely due to the inability of the former to form true hyphae. Furthermore, *C. albicans* and other non-*C. albicans Candida* species adhere to a greater extent to human mucosae than *C. parapsilosis* (166, 179). Adherence to mucosal surfaces is an essential prerequisite of *C. albicans* and other pathogenic *Candida* species to colonize and subsequently infect mucosal tissues. Slime production and biofilm formation are potentially significant virulence factors of *C. parapsilosis* enabling adhesion to plastic medical devices such as intravascular catheters (80, 99, 162, 166). *Candida parapsilosis* readily forms biofilms when grown in media containing high lipid and glucose concentrations, which reflects the increased prevalence of bloodstream infections caused by this organism in patents receiving parenteral nutrition (99).

A variety of typing techniques have shown that strains considered to be *C. parapsilosis* are genotypically heterogenous and can be subdivided into three distinct groups (87, 89, 99, 127, 128, 159). Group I strains predominate in clinical samples, whereas groups II and III account for approximately 10% of isolates (87, 160). Data from the analysis of distinct molecular markers and multilocus sequence typing resulted in the classification of the three groups as distinct species: *C. parapsilosis* (group I), *C. orthopsilosis* (group II), and *C. metapsilosis* (group III) (159). *Candida parapsilosis* isolates exhibit significantly lower nucleotide sequence diversity than *C. orthopsilosis* and *C. metapsilosis*, suggesting that the species may have diverged relatively recently (166). Whereas biofilm formation is a feature of *C. parapsilosis* isolates, it is uncommon among isolates of *C. orthopsilosis* and *C. metapsilosis*, which may contribute to the lower prevalence of these two species among clinical isolates (141, 159, 160). *Candida parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* belong to the same branch in the phylogenetic tree with the CTG clade of hemiascomycetes along with other clinically important pathogenic yeasts, including *C. albicans*, *C. dubliniensis*, and *C. tropicalis* (42, 74, 99).

Candida parapsilosis isolates are generally susceptible to commonly used systemic antifungal drugs, including azoles (fluconazole, ketoconazole, itraconazole, voriconazole, and posaconazole) and polyenes (amphotericin B) (104, 111, 117, 118, 166). Reduced susceptibility and resistance to azoles have been reported for *C. parapsilosis*, although they are relatively rare (131). Furthermore, tolerance and resistance to amphotericin B have also been reported (133). *C. parapsilosis* has been associated with a higher MIC of echinocandin antifungal drugs relative to other *Candida* species (166). *C. parapsilosis* sensu stricto (formerly *C. parapsilosis* group I) has been reported to have significantly higher caspofungin and anidulafungin MICs than *C. orthopsilosis* and *C. metapsilosis* (165).

Candida tropicalis

Candida tropicalis grows as oval yeast cells and as pseudohyphae. It is a diploid organism and belongs to the same branch in the phylogenetic tree as the CTG clade of hemiascomycetes along with other clinically important pathogenic yeasts, including *C. albicans*, *C. dubliniensis*, *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis*. *Candida tropicalis* can be found as part of the normal commensal flora in humans and can cause invasive candidiasis in patients with severe underlying disease, especially in patients with cancer (71, 79, 124, 136, 173). Indeed, *C. tropicalis* has a tendency to cause disseminated infection in neutropenic patients and is responsible for a disproportionate number of severe infections in patients with hematologic malignancies (179). Most infections appear to be endogenously acquired from the normal commensal flora, most likely from the intestinal tract, although nosocomial acquisition can occur (109, 124, 125, 176). *Candida tropicalis* is rarely associated with oropharyngeal infection and appears to be more virulent than *C. albicans* in patients with hematologic malignancies, and disseminated infection is associated with high mortality rates (73, 79, 173, 176).

Whereas *C. albicans* is still the leading cause of candidemia in most centers, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* comprise the three most frequently isolated non-*C. albicans Candida* species from bloodstream infections in most hospitals, although differences in their distribution have been reported from different countries (8, 15, 27, 35, 79,

103, 109, 112, 124, 136, 173). In India *C. tropicalis* is more commonly isolated than either of the other two non-*C. albicans Candida* species from both adult and pediatric patients (27, 135, 172).

Candida tropicalis isolates are susceptible to commonly used systemic antifungal drugs, including azoles (fluconazole, ketoconazole, itraconazole, voriconazole, and posaconazole), polyenes (amphotericin B), and echinocandins. Resistance to azoles (particularly fluconazole) and polyenes has been reported for clinical isolates, and more recently resistance to caspofungin has been reported (15, 35, 36, 71, 83, 107, 110, 168, 177).

CANDIDA SPECIES INFREQUENTLY ASSOCIATED WITH HUMAN DISEASE

Candida krusei

Candida krusei grows as yeasts and pseudohyphae and is the anamorphic form of *Issatchenkia orientalis* (39). In comparison with the previously described species, *C. krusei* is relatively rarely associated with infection and is responsible for approximately 3% of cases of IC; it is most often associated with patients suffering from hematologic malignancies (1, 2). As it is a member of the gastrointestinal microflora, most infections are believed to be endogenous in origin (73). *Candida krusei* is of particular note because of its multidrug resistance profile due to inherent reduced susceptibility to the commonly used antifungal agents fluconazole and amphotericin B (113). However, it is susceptible to newly developed azole drugs and caspofungin, although there has been a report of caspofungin failure in a case of disseminated *C. krusei* candidiasis (108). While exposure of HIV patients to fluconazole may have led to the emergence of this species as a significant cause of recurrent OPC (84), surveillance studies suggest that the incidence of *C. krusei* IC has remained constant, and it has not emerged as a significant cause of disease despite the widespread use of fluconazole (112).

Candida dubliniensis

Candida dubliniensis was first described in cases of OPC in HIV-infected and AIDS patients in 1995 (157). It is very closely related to *C. albicans* and has the ability to grow as yeasts, pseudohyphae, hyphae, and chlamydospores (153). Given the close relationship between the two species, *C. albicans* and *C. dubliniensis* are phenotypically very similar and it is often difficult to differentiate between them in clinical samples. The simplest phenotypic tests include differential growth at 42°C and in hypertonic media and colony morphology on Pal's or Staib agar (5–7, 120). However, PCR-based tests are still the most accurate means of identifying *C. dubliniensis* (46, 126, 155). Despite the close relationship of *C. albicans* and *C. dubliniensis*, the latter is a rare cause of IC (approximately 2 to 5% of cases [11, 29, 69, 103]). The predisposing factors and the crude mortality associated with infection have been shown to be similar for the two species (29). *Candida dubliniensis* is a minor constituent of the normal oral flora of healthy individuals and is primarily associated with OPC, particularly in HIV-infected individuals (152, 154). Although *C. dubliniensis* has the capacity to develop azole resistance in vitro (96), clinical resistance to antifungals is rare (113).

Candida guilliermondii

Candida guilliermondii grows as a yeast and as pseudohyphae and is the anamorphic state of *Pichia guilliermondii* (39). It is most often associated with nail infections (54); however, it is a rare cause of invasive infection (usually <1% of cases) (115). The incidence of *C. guilliermondii* cases of IC is somewhat higher in Latin America than in other parts of the world, and in a recent study from India it was isolated from 30% of pediatric patients in a single tertiary care center, suggesting an exogenous source of infection and possible infection control deficiencies (27). Therefore, although *C. guilliermondii* infections may occur independently, nosocomial clusters (such as that in India described above) have also been recorded, and infection may be related to the usage of intravenous catheters (27, 93). There is evidence for reduced susceptibility to fluconazole in skin isolates; however, blood isolates tend to be susceptible to this and other antifungal agents (115).

Candida lusitaniae

Candida lusitaniae grows in yeast and pseudohyphal forms and is the anamorphic state of *Clavispora lusitaniae* (39). It is only rarely associated with disease; however, it is noteworthy due to reports of resistance to amphotericin B among some clinical isolates (14, 105). The incidence of *C. lusitaniae* infections was very low before the introduction of aggressive chemotherapeutic treatments; however, it increased dramatically, particularly in cancer patients, after 1990 (73). Although there is some evidence for amphotericin B resistance in *C. lusitaniae*, it is highly susceptible to azoles and echinocandins, and fluconazole is recommended as the most suitable treatment (174).

Candida rugosa

Candida rugosa, as with many other non-*C. albicans Candida* species, grows by vegetative budding and produces pseudohyphae, but it has no known teleomorphic form (39). It is primarily known as a cause of bovine mastitis (34) and has rarely been associated with human disease. Only 27 cases of *C. rugosa* candidemia have been reported in the literature (95), with most of these associated with two outbreaks (33, 47). However, this species deserves attention due to reduced susceptibility to azole and other antifungal agents (114) and to the apparent clustering of infections, suggesting an environmental or nosocomial source of infection.

OTHER *CANDIDA* SPECIES ASSOCIATED WITH HUMAN DISEASE

The remaining *Candida* species associated with human disease are only rarely detected, and therefore relatively little is known about the etiology or the epidemiology of the diseases they cause. These species include *C. famata*, *C. inconspicua*, *C. kefyr*, *C. lipolytica*, *C. norvegensis*, *C. sake*, and *C. zeylanoides*, as well as newly described species, such as *C. bracarensis* and *C. nivariensis*. Although rare, clinical isolates belonging to several of these species are resistant to one or more antifungal drugs; thus, they have the potential to emerge as more significant pathogens.

CONCLUDING REMARKS

Despite the dramatic reduction in the incidence of OPC in HIV/AIDS patients (due to HAART) during the last decade or so, *Candida* species continue to be the most important human fungal pathogens. However, despite improvements in the therapy and diagnosis of IC over this period, the incidence, morbidity, and mortality associated with IC remain high. Although the reasons for this are likely to be complex,

there are ever-increasing numbers of patients with risk factors for acquiring disease (e.g., an aging population, more patients with severe underlying disease being hospitalized for longer periods and requiring catheterization, etc.). Similar reasons may also help to explain shifts in the species responsible for cases of IC observed in certain parts of the world. The more medically compromised a patient is, the higher his or her risk of being infected by less pathogenic species. Another reason for the increasing reports of rare species is that clinicians are increasingly aware of the potential importance of these species and that routine diagnostic laboratories are now better able to rapidly and accurately identify them. However, the role of the increased usage of antifungal agents, such as fluconazole, in the selection of species with intrinsic resistance or species with an enhanced capacity to develop resistance on exposure to the drug cannot be discounted. For these reasons patients receiving prophylactic treatment designed to prevent IC should be monitored carefully. It is evident that many cases of IC, particularly those caused by species such as *C. parapsilosis*, are acquired due to lapses in infection control and prevention. Clearly, in these instances improved hygiene and catheter usage could help to lower the incidence of infection. Finally, since the vast majority of isolates recovered from cases of IC are susceptible to antifungal agents, it is evident that the faster these drugs are administered to patients, the greater their chance of survival will be. While there have been improvements in the development of diagnostic tests recently, there is certainly room for improvement, and it is hoped that ongoing research into novel methods will ultimately lead to the development of more rapid and accurate diagnosis of *Candida* infections.

REFERENCES

1. **Abbas, J., G. P. Bodey, H. A. Hanna, M. Mardani, E. Girgawy, D. Abi-Said, E. Whimbey, R. Hachem, and I. Raad.** 2000. *Candida krusei* fungemia: an escalating serious infection in immunocompromised hosts. *Arch. Intern. Med.* **160:**2659–2664.
2. **Abi-Said, D., E. Anaissie, O. Uzun, I. Raad, H. Pinzcowski, and S. Vartivarian.** 1997. The epidemiology of hematogenous candidiasis caused by different *Candida* species. *Clin. Infect. Dis.* **24:**1122–1128.
3. **Akisada, T., K. Harada, M. Niimi, and A. Kamaguchi.** 1983. Production of contiguously arranged chlamydospores in *Candida albicans*. *J. Gen. Microbiol.* **129:**2327–2330.
4. **Almeida, R. S., S. Brunke, A. Albrecht, S. Thewes, M. Laue, J. E. Edwards, S. G. Filler, and B. Hube.** 2008. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog.* **4:**e1000217.
5. **Al Mosaid, A., D. Sullivan, I. F. Salkin, D. Shanley, and D. C. Coleman.** 2001. Differentiation of *Candida dubliniensis* from *Candida albicans* on Staib agar and caffeic acid-ferric citrate agar. *J. Clin. Microbiol.* **39:**323–327.
6. **Al-Mosaid, A., D. J. Sullivan, and D. C. Coleman.** 2003. Differentiation of *Candida dubliniensis* from *Candida albicans* on Pal's agar. *J. Clin. Microbiol.* **41:**4787–4789.
7. **Alves, S. H., E. P. Milan, P. de Laet Sant'Ana, L. O. Oliveira, J. M. Santurio, and A. L. Colombo.** 2002. Hypertonic Sabouraud broth as a simple and powerful test for *Candida dubliniensis* screening. *Diagn. Microbiol. Infect. Dis.* **43:**85–86.
8. **Anunnatsiri, S., P. Chetchotisakd, and P. Mootsikapun.** 2009. Fungemia in non-HIV-infected patients: a five-year review. *Int. J. Infect. Dis.* **13:**90–96.
9. **Arendrup, M., T. Horn, and N. Frimodt-Møller.** 2002. In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. *Infection* **30:**286–291.
10. **Arribas, J. R., S. Hernández-Albujar, J. J. González-García, J. M. Peña, A. Gonzalez, T. Cañedo, R. Madero, J. J. Vazquez, and W. G. Powderly.** 2000. Impact of protease inhibitor therapy on HIV-related oropharyngeal candidiasis. *AIDS* **14:**979–985.
11. **Ásmundsdóttir, L. R., H. Erlendsdóttir, G. Haraldsson, H. Guo, J. Xu, and M. Gottfredsson.** 2008. Molecular epidemiology of candidemia: evidence of clusters of smoldering nosocomial infections. *Clin. Infect. Dis.* **47:** e17–e24.
12. **Bagg, J., M. P. Sweeney, M. A. Lewis, M. S. Jackson, D. Coleman, A. Al Mosaid, W. Baxter, S. McEndrick, and S. McHugh.** 2003. High prevalence of non-*albicans* yeasts and detection of anti-fungal resistance in the oral flora of patients with advanced cancer. *Palliat. Med.* **17:**477–481.
13. **Bennett, J. E., K. Izumikawa, and K. A. Marr.** 2004. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. *Antimicrob. Agents Chemother.* **48:**1773–1777.
14. **Blinkhorn, R. J., D. Adelstein, and P. J. Spagnuolo.** 1989. Emergence of a new opportunistic pathogen, *Candida lusitaniae*. *J. Clin. Microbiol.* **27:**236–240.
15. **Borg-von Zepelin, M., L. Kunz, R. Ruchel, U. Reichard, M. Weig, and U. Gross.** 2007. Epidemiology and antifungal susceptibilities of *Candida* spp. to six antifungal agents: results from a surveillance study on fungaemia in Germany from July 2004 to August 2005. *J. Antimicrob. Chemother.* **60:**424–428.
16. **Bougnoux, M. E., D. Diogo, N. Francois, B. Sendid, S. Veirmeire, J. F. Colombel, C. Bouchier, H. Van Kruiningen, C. d'Enfert, and D. Poulain.** 2006. Multilocus sequence typing reveals intrafamilial transmission and microevolutions of *Candida albicans* isolates from the human digestive tract. *J. Clin. Microbiol.* **44:**1810–1820.
17. **Bougnoux, M. E., C. Pujol, D. Diogo, C. Bouchier, D. R. Soll, and C. d'Enfert.** 2008. Mating is rare within as well as between clades of the human pathogen *Candida albicans*. *Fungal Genet. Biol.* **45:**221–231.
18. **Brockert, P. J., S. A. Lachke, T. Srikantha, C. Pujol, R. Galask, and D. R. Soll.** 2003. Phenotypic switching and mating type switching of *Candida glabrata* at sites of colonization. *Infect. Immun.* **71:**7109–7118.
19. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. S. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J. L. Reedy, I. Agrafioti, M. B. Arnaud, S. Bates, A. J. P. Brown, S. Brunke, M. C. Costanzo, D. A. Fitzpatrick, P. W. J. de Groot, D. Harris, L. L. Hoyer, B. Hube, F. M. Klis, C. Kodira, N. Lennard, M. E. Logue, R. Martin, A. M. Neiman, E. Nikolaou, M. A. Quail, J. Quinn, M. C. Santos, F. F. Schmitzberger, G. Sherlock, P. Shah, K. A. T. Silverstein, M. S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M. P. H. Stumpf, P. E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N. A. R. Gow, M. C. Lorenz, B. W. Birren, M. Kellis, and C. A. Cuomo.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* 459:657–662.
20. **Calderone, R. A.** 2002. Introduction and historical perspectives, p. 3–13. *In* R. A. Calderone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
21. **Campbell, J. R., E. Zaccaria, and C. J. Baker.** 2000. Systemic candidiasis in extremely low birth weight infants receiving topical petrolatum ointment for skin care: a case-control study. *Pediatrics* **105:**1041–1045.
22. **Cano, M. V., J. F. Perz, A. S. Craig, M. Liu, G. M. Lyon, M. E. Brandt, T. J. Lott, B. A. Lasker, F. F. Barrett,**

M. M. McNeil, W. Schaffner, and R. A. Hajjeh. 2005. Candidemia in pediatric outpatients receiving home total parenteral nutrition. *Med. Mycol.* **43:**219–225.
23. **Carruba, G., E. Pontieri, F. De Bernardis, P. Martino, and A. Cassone.** 1991. DNA fingerprinting and electrophoretic karyotypes of environmental and clinical isolates of *Candida parapsilosis*. *J. Clin. Microbiol.* **29:**916–922.
24. **Cassone, A., F. De Bernardis, E. Pontieri, G. Carruba, C. Girmenia, P. Martino, M. Fernandez-Rodriguez, G. Quindos, and J. Ponton.** 1995. Biotype diversity of *Candida parapsilosis* and its relationship to the clinical source and experimental pathogenicity. *J. Infect. Dis.* **171:**967–975.
25. **Cassone, A., F. De Bernardis, A. Torosantucci, E. Tacconelli, M. Tumbarello, and R. Cauda.** 1999. In vitro and in vivo anticandidal activity of human immunodeficiency virus protease inhibitors. *J. Infect. Dis.* **180:**448–453.
26. **Cauda, R., E. Tacconelli, M. Tumbarello, G. Morace, F. De Bernardis, A. Torosantucci, and A. Cassone.** 1999. Role of protease inhibitors in preventing recurrent oral candidosis in patients with HIV infection: a prospective case-control study. *J Acquir. Immune Defic. Syndr.* **21:**20–25.
27. **Chakrabarti, A., S. S. Chatterjee, K. L. N. Rao, M. M. Zameer, M. R. Shivaprakash, S. Singhi, R. Singh, and S. C. Varma.** 2009. Recent experience with fungaemia: change in species distribution and azole resistance. *Scand. J. Infect. Dis.* **41:**275–284.
28. **Chapeland-Leclerc, F., C. Hennequin, N. Papon, T. Noël, A. Girard, G. Socié, P. Ribaud, and C. Lacroix.** 2010. Acquisition of flucytosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. *Antimicrob. Agents Chemother.* **54:**1360–1362.
29. **Chen, S. C. A., D. Marriott, E. G. Playford, Q. Nguyen, D. Ellis, W. Meyer, T. C. Sorrell, and M. Slavin.** 2009. Candidaemia with uncommon *Candida* species: predisposing factors, outcome, antifungal susceptibility, and implications for management. *Clin. Microbiol. Infect.* **15:**662–669.
30. **Citiulo, F., G. P. Moran, D. C. Coleman, and D. J. Sullivan.** 2009. Purification and germination of *Candida albicans* and *Candida dubliniensis* chlamydospores cultured in liquid media. *FEMS Yeast Res.* **9:**1051–1060.
31. **Clayton, Y. M., and W. C. Noble.** 1966. Observations on the epidemiology of *Candida albicans*. *J. Clin. Pathol.* **19:**76–78.
32. **Coleman, D. C., D. E. Bennett, D. J. Sullivan, P. J. Gallagher, M. C. Henman, D. B. Shanley, and R. J. Russell.** 1993. Oral *Candida* in HIV infection and AIDS: new perspectives/new approaches. *Crit. Rev. Microbiol.* 19:61–82.
33. **Colombo, A. L., A. S. Azevedo Melo, R. F. Crespo Rosas, R. Salomão, M. Briones, R. J. Hollis, S. A. Messer, and M. A. Pfaller.** 2003. Outbreak of *Candida rugosa* candidemia: an emerging pathogen that may be refractory to amphotericin B therapy. *Diagn. Microbiol. Infect. Dis.* **46:**253–257.
34. **Crawshaw, W. M., N. R. MacDonald, and G. Duncan.** 2005. Outbreak of *Candida rugosa* mastitis in a dairy herd after intramammary antibiotic treatment. *Vet. Rec.* **156:**812–813.
35. **Cuenca-Estrella, M., D. Rodriguez, B. Almirante, J. Morgan, A. M. Planes, M. Almela, J. Mensa, F. Sanchez, J. Ayats, M. Gimenez, M. Salvado, D. W. Warnock, A. Pahissa, and J. L. Rodriguez-Tudela.** 2005. In vitro susceptibilities of bloodstream isolates of *Candida* species to six antifungal agents: results from a population-based active surveillance programme, Barcelona, Spain, 2002–2003. *J. Antimicrob. Chemother.* **55:**194–199.
36. **da Costa, K. R., J. C. Ferreira, M. C. Komesu, and R. C. Candido.** 2009. *Candida albicans* and *Candida tropicalis* in oral candidosis: quantitative analysis, exoenzyme activity, and antifungal drug sensitivity. *Mycopathologia* **167:**73–79.
37. **da Silva, C. L., R. M. dos Santos, and A. L. Colombo.** 2001. Cluster of *Candida parapsilosis* primary bloodstream infections in neonatal intensive care unit. *Brazil. J. Infect. Dis.* **171:**32–36.
38. **Davies, A. N., S. Brailsford, K. Broadley, and D. Beighton.** 2002. Oral yeast carriage in patients with advanced cancer. *Oral Microbiol. Immunol.* **17:**79–84.
39. **de Hoog, G. S., and J. Guarro.** 1995. *Candida krusei*, p. 228. *In* G. S. de Hoog and J. Guarro (ed.), *Atlas of Clinical Fungi*. Centraalbureau voor Schimmelcultures/Universitat Rovira i Virgili, Utrecht, The Netherlands.
40. **de Repentigny, L., F. Aumont, K. Bernard, and P. Belhumeur.** 2000. Characterization of binding of *Candida albicans* to small intestinal mucin and its role in adherence to mucosal epithelial cells. *Infect. Immun.* **68:**3172–3179.
41. **Diekema, D. J., S. A. Messer, A. B. Brueggemann, S. L. Coffman, G. V. Doern, L. A. Herwaldt, and M. A. Pfaller.** 2002. Epidemiology of candidemia: 3-year results from the Emerging Infections and the Epidemiology of Iowa Organisms study. *J. Clin. Microbiol.* **40:**1298–1302.
42. **Diezmann, S., C. J. Cox, G. Schonian, R. J. Vilgalys, and T. G. Mitchell.** 2004. Phylogeny and evolution of medical species of *Candida* and related taxa: a multigenic analysis. *J. Clin. Microbiol.* **42:**5624–5635.
43. **Dodgson, A. R., K. J. Dodgson, C. Pujol, M. A. Pfaller, and D. R. Soll.** 2004. Clade-specific flucytosine resistance is due to a single nucleotide change in the *FUR1* gene of *Candida albicans*. *Antimicrob. Agents Chemother.* **48:**2223–2227.
44. **Domergue, R., I. Castaño, A. De Las Peñas, M. Zupancic, V. Lockatell, J. R. Hebel, D. Johnson, and B. P. Cormack.** 2005. Nicotinic acid limitation regulates silencing of *Candida* adhesins during UTI. *Science* **308:**866–870.
45. **Dongari-Bagtzoglou, A., P. Dwivedi, E. Ioannidou, M. Shaqman, D. Hull, and J. Burleson.** 2009. Oral *Candida* infection and colonization in solid organ transplant recipients. *Oral Microbiol. Immunol.* **24:**249–254.
46. **Donnelly, S. A., D. J. Sullivan, D. B. Shanley, and D. C. Coleman.** 1999. Phylogenetic analysis and rapid identification of *Candida dubliniensis* based on analysis of *ACT1* intron and exon sequences. *Microbiology* **145:**1871–1882.
47. **Dube, M. P., P. N. Heseltine, M. G. Rinaldi, S. Evans, and B. Zawacki.** 1994. Fungemia and colonization with nystatin-resistant *Candida rugosa* in a burn unit. *Clin. Infect. Dis.* **18:**77–82.
48. **Felk, A., M. Kretschmar, A. Albrecht, M. Schaller, S. Beinhauer, T. Nichterlein, D. Sanglard, H. C. Korting, W. Schafer, and B. Hube.** 2002. *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infect. Immun.* **70:**3689–3700.
49. **Fidel, P. L., J. A. Vazquez, and J. D. Sobel.** 1999. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin. Microbiol. Rev.* **12:**80–96.
50. **Fitzpatrick, D., M. Logue, J. Stajich, and G. Butler.** 2006. A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. *BMC Evol. Biol.* **6:**99.
51. **Forche, A., K. Alby, D. Schaefer, A. D. Johnson, J. Berman, and R. J. Bennett.** 2008. The parasexual cycle in *Candida albicans* provides an alternative pathway to meiosis for the formation of recombinant strains. *PLoS Biol.* **6:**e110.
52. **Gadanho, M., and J. Sampaio.** 2005. Occurrence and diversity of yeasts in the Mid-Atlantic Ridge hydrothermal fields near the Azores archipelago. *Microb. Ecol.* **50:**408–417.

53. **Garcia-Effron, G., S. Lee, S. Park, J. D. Cleary, and D. S. Perlin.** 2009. Effect of *Candida glabrata FKS1* and *FKS2* mutations on echinocandin sensitivity and kinetics of 1,3-β-D-glucan synthase: implication for the existing susceptibility breakpoint. *Antimicrob. Agents Chemother.* **53:**3690–3699.
54. **Ghannoum, M. A., R. A. Hajjeh, R. Scher, N. Konnikov, A. K. Gupta, R. Summerbell, S. Sullivan, R. Daniel, P. Krusinski, P. Fleckman, P. Rich, R. Odom, R. Aly, D. Pariser, M. Zaiac, G. Rebell, J. Lesher, B. Gerlach, G. F. Ponce de Leon, A. Ghannoum, J. Warner, N. Isham, and B. Elewski.** 2000. A large-scale North American study of fungal isolates from nails: the frequency of onychomycosis, fungal distribution, and antifungal susceptibility patterns. *J. Am. Acad. Dermatol.* **43:**641–648.
55. **Gruber, A., C. Speth, E. Lukasser-Vogl, R. Zangerle, M. Borg-von Zepelin, M. P. Dierich, and R. Würzner.** 1999. Human immunodeficiency virus type 1 protease inhibitor attenuates *Candida albicans* virulence properties in vitro. *Immunopharmacology* **41:**227–234.
56. **Gudlaugsson, O., S. Gillespie, K. Lee, J. Vande Berg, J. Hu, S. Messer, L. Herwaldt, M. Pfaller, and D. Diekema.** 2003. Attributable mortality of nosocomial candidemia, revisited. *Clin. Infect. Dis.* **37:**1172–1177.
57. **Hachem, R., H. Hanna, D. Kontoyiannis, Y. Jiang, and I. Raad.** 2008. The changing epidemiology of invasive candidiasis: *Candida glabrata* and *Candida krusei* as the leading causes of candidemia in hematologic malignancy. *Cancer* **112:**2493–2499.
58. **Henry, K. W., J. T. Nickels, and T. D. Edlind.** 2000. Upregulation of *ERG* genes in *Candida* species by azoles and other sterol biosynthesis inhibitors. *Antimicrob. Agents Chemother.* **44:**2693–2700.
59. **Hoyer, L. L., T. L. Payne, M. Bell, A. M. Myers, and S. Scherer.** 1998. *Candida albicans ALS3* and insights into the nature of the *ALS* gene family. *Curr. Genet.* **33:**451–459.
60. **Huang, Y. C., C. C. Li, T. Y. Lin, R. I. Lien, Y. H. Chou, J. L. Wu, and C. Hsueh.** 1998. Association of fungal colonization and invasive disease in very low birth weight infants. *Pediatr. Infect. Dis. J.* **17:**819–822.
61. **Huang, Y. C., T. Y. Lin, H. S. Leu, H. L. Peng, J. H. Wu, and H. Y. Chang.** 1999. Outbreak of *Candida parapsilosis* fungemia in neonatal intensive care units: clinical implications and genotyping analysis. *Infection* **27:**97–102.
62. **Hull, C. M., and A. D. Johnson.** 1999. Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. *Science* **285:**1271–1275.
63. **Hull, C. M., R. M. Raisner, and A. D. Johnson.** 2000. Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. *Science* **289:**307–310.
64. **Jackson, A. P., J. A. Gamble, T. Yeomans, G. P. Moran, D. Saunders, D. Harris, M. Aslett, J. F. Barrell, G. Butler, F. Citiulo, D. C. Coleman, P. W. de Groot, T. J. Goodwin, M. A. Quail, J. McQuillan, C. A. Munro, A. Pain, R. T. Poulter, M. A. Rajandream, H. Renauld, M. J. Spiering, A. Tivey, N. A. Gow, B. Barrell, D. J. Sullivan, and M. Berriman.** 2009. Comparative genomics of the fungal pathogens *Candida dubliniensis* and *C. albicans*. *Genome Res.* **10:**10.
65. **Kadir, T., R. Pisiriciler, S. Akyüz, A. Yarat, N. Emekli, and A. Ipbüker.** 2002. Mycological and cytological examination of oral candidal carriage in diabetic patients and non-diabetic control subjects: thorough analysis of local aetiologic and systemic factors. *J. Oral Rehabil.* **29:**452–457.
66. **Kaur, R., I. Castaño, and B. P. Cormack.** 2004. Functional genomic analysis of fluconazole susceptibility in the pathogenic yeast *Candida glabrata*: roles of calcium signaling and mitochondria. *Antimicrob. Agents Chemother.* **48:**1600–1613.
67. **Kent, H. L.** 1991. Epidemiology of vaginitis. *Am. J. Obstet. Gynecol.* **165:**1168–1176.
68. **Khan, Z. U., S. Ahmad, I. Al-Obaid, N. A. Al-Sweih, L. Joseph, and D. Farhat.** 2008. Emergence of resistance to amphotericin B and triazoles in *Candida glabrata* vaginal isolates in a case of recurrent vaginitis. *J. Chemother.* **20:**488–491.
69. **Kibbler, C. C., S. Ainscough, R. A. Barnes, W. R. Gransden, R. E. Holliman, E. M. Johnson, J. D. Perry, D. J. Sullivan, and J. A. Wilson.** 2003. Management and outcome of blood stream infections due to *Candida* species in England and Wales. *J. Hosp. Infect.* **54:**18–24.
70. **King, R. D., J. C. Lee, and A. L. Morris.** 1980. Adherence of *Candida albicans* and other *Candida* species to mucosal epithelial cells. *Infect. Immun.* **27:**667–674.
71. **Kofteridis, D. P., R. E. Lewis, and D. P. Kontoyiannis.** 2010. Caspofungin-non-susceptible *Candida* isolates in cancer patients. *J. Antimicrob. Chemother.* **65:**293–295.
72. **Koh, A. Y., J. R. Köhler, K. T. Coggshall, N. Van Rooijen, and G. B. Pier.** 2008. Mucosal damage and neutropenia are required for *Candida albicans* dissemination. *PLoS Pathog.* **4:**e35.
73. **Krcmery, V., and A. J. Barnes.** 2002. Non-*albicans Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. *J. Hosp. Infect.* **50:**243–260.
74. **Kurtzman, C. P., and C. J. Robnett.** 1997. Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 5′ end of the large-subunit (26S) ribosomal DNA gene. *J. Clin. Microbiol.* **35:**1216–1223.
75. **Lass-Flörl, C.** 2009. The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* **52:**197–205.
76. **Lee, I., N. O. Fishman, T. E. Zaoutis, K. H. Morales, M. G. Weiner, M. Synnestvedt, I. Nachamkin, and E. Lautenbach.** 2009. Risk factors for fluconazole-resistant *Candida glabrata* bloodstream infections. *Arch. Intern. Med.* **169:**379–383.
77. **Lermann, U., and J. Morschhaüser.** 2008. Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*. *Microbiology* **154:**3281–3295.
78. **Leroy, O., J. P. Gangneux, P. Montravers, J. P. Mira, F. Gouin, J. P. Sollet, J. Carlet, J. Reynes, M. Rosenheim, B. Regnier, and O. Lortholary.** 2009. Epidemiology, management, and risk factors for death of invasive *Candida* infections in critical care: a multicenter, prospective, observational study in France (2005–2006). *Crit. Care Med.* **37:**1612–1618.
79. **Leung, A. Y., C. S. Chim, P. L. Ho, V. C. Cheng, K. Y. Yuen, A. K. Lie, W. Y. Au, R. Liang, and Y. L. Kwong.** 2002. *Candida tropicalis* fungaemia in adult patients with haematological malignancies: clinical features and risk factors. *J. Hosp. Infect.* **50:**316–319.
80. **Levin, A. S., S. F. Costa, N. S. Mussi, M. Basso, S. I. Sinto, C. Machado, D. C. Geiger, M. C. Villares, A. Z. Schreiber, A. A. Barone, and M. L. Branchini.** 1998. *Candida parapsilosis* fungemia associated with implantable and semi-implantable central venous catheters and the hands of healthcare workers. *Diagn. Microbiol. Infect. Dis.* **30:**243–249.
81. **Levy, I., L. G. Rubin, S. Vasishtha, V. Tucci, and S. K. Sood.** 1998. Emergence of *Candida parapsilosis* as the predominant species causing candidemia in children. *Clin. Infect. Dis.* **26:**1086–1088.
82. **Li, L., S. Redding, and A. Dongari-Bagtzoglou.** 2007. *Candida glabrata*: an emerging oral opportunistic pathogen. *J. Dent. Res.* **86:**204–215.
83. **Li, S. Y., Y. L. Yang, Y. H. Lin, H. C. Ho, A. H. Wang, K. W. Chen, C. W. Wang, H. Chi, and H. J. Lo.** 2009. Two closely related fluconazole-resistant *Candida tropicalis*

clones circulating in Taiwan from 1999 to 2006. *Microb. Drug Resist.* **15:**205–210.
84. **Lischewski, A., M. Rhunke, I. Tennagen, G. Schönian, J. Morschhäuser, and J. Hacker.** 1995. Molecular epidemiology of *Candida* isolates from AIDS patients showing different fluconazole resistance profiles. *J. Clin. Microbiol.* **33:**769–771.
85. **Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90:**939–949.
86. **Lockhart, S. R., S. Joly, K. Vargas, J. Swails-Wenger, L. Enger, and D. R. Soll.** 1999. Natural defenses against *Candida* colonization breakdown in the oral cavities of the elderly. *J. Dent. Res.* **78:**857–868.
87. **Lockhart, S. R., S. A. Messer, M. A. Pfaller, and D. J. Diekema.** 2008. *Lodderomyces elongisporus* masquerading as *Candida parapsilosis* as a cause of bloodstream infections. *J. Clin. Microbiol.* **46:**374–376.
88. **Lockhart, S. R., B. D. Reed, C. L. Pierson, and D. R. Soll.** 1996. Most frequent scenario for recurrent *Candida* vaginitis is strain maintenance with "substrain shuffling": demonstration by sequential DNA fingerprinting with probes Ca3, C1, and CARE2. *J. Clin. Microbiol.* **34:**767–777.
89. **Lott, T. J., R. J. Kuykendall, S. F. Welbel, A. Pramanik, and B. A. Lasker.** 1993. Genetic heterogeneity in the yeast *Candida parapsilosis*. *Curr. Genet.* **23:**463–467.
90. **Luo, G., and L. P. Samaranayake.** 2002. *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. *APMIS* **110:**601–610.
91. **Magee, B. B., and P. T. Magee.** 2000. Induction of mating in *Candida albicans* by construction of *MTL*a and *MTL*α strains. *Science* **289:**310–313.
92. **Marais, E., R. Stewart, A. G. Duse, I. C. Rosekilly, G. de Jong, and N. Aithma.** 2004. *Candida parapsilosis* detected in TPN using the BacT/Alert system and characterized by randomly amplified polymorphic DNA. *J. Hosp. Infect.* **56:**291–296.
93. **Masala, L., R. Luzzati, L. Maccacaro, L. Antozzi, E. Concia, and R. Fontana.** 2003. Nosocomial cluster of *Candida guillermondii* fungemia in surgical patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **22:**686–688.
94. **Medeiros, A. O., L. M. Kohler, J. S. Hamdan, B. S. Missagia, F. A. Barbosa, and C. A. Rosa.** 2008. Diversity and antifungal susceptibility of yeasts from tropical freshwater environments in Southeastern Brazil. *Water Res.* **42:**3921–3929.
95. **Minces, L. R., K. S. Ho, P. J. Veldkamp, and C. J. Clancy.** 2009. *Candida rugosa*: a distinctive emerging cause of candidaemia. A case report and review of the literature. *Scand. J. Infect. Dis.* **41:**892–897.
96. **Moran, G. P., D. J. Sullivan, M. C. Henman, C. E. McCreary, B. J. Harrington, D. B. Shanley, and D. C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. *Antimicrob. Agents Chemother.* **41:**617–623.
97. **Morschhäuser, J.** 2010. Regulation of multidrug resistance in pathogenic fungi. *Fungal Genet. Biol.* **94:**94–106.
98. **Naglik, J. R., S. J. Challacombe, and B. Hube.** 2003. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev.* **67:**400–428.
99. **Nosek, J., Z. Holesova, P. Kosa, A. Gacser, and L. Tomaska.** 2009. Biology and genetics of the pathogenic yeast *Candida parapsilosis*. *Curr. Genet.* **55:**497–509.
100. **Odds, F. C.** 1988. Candida *and Candidosis: a Review and Bibliography*. Bailliere Tindall, London, United Kingdom.
101. **Odds, F. C., A. D. Davidson, M. D. Jacobsen, A. Tavanti, J. A. Whyte, C. C. Kibbler, D. H. Ellis, M. C. J. Maiden, D. J. Shaw, and N. A. R. Gow.** 2006. *Candida albicans* strain maintenance, replacement, and microvariation demonstrated by multilocus sequence typing. *J. Clin. Microbiol.* **44:**3647–3658.
102. **Odds, F. C., N. A. R. Gow, and A. J. P. Brown.** 2001. Fungal virulence studies come of age. *Genome Biol.* **2:**reviews1009.1–reviews1009.4.
103. **Odds, F. C., M. F. Hanson, A. D. Davidson, M. D. Jacobsen, P. Wright, J. A. Whyte, N. A. R. Gow, and B. L. Jones.** 2007. One year prospective survey of *Candida* bloodstream infections in Scotland. *J. Med. Microbiol.* **56:**1066–1075.
104. **Ostrosky-Zeichner, L., B. D. Alexander, D. H. Kett, J. Vazquez, P. G. Pappas, F. Saeki, P. A. Ketchum, J. Wingard, R. Schiff, H. Tamura, M. A. Finkelman, and J. H. Rex.** 2005. Multicenter clinical evaluation of the (1→3) β-D-glucan assay as an aid to diagnosis of fungal infections in humans. *Clin. Infect. Dis.* **41:**654–659.
105. **Pappagianis, D., M. S. Collins, R. Hector, and J. Remington.** 1979. Development of resistance to amphotericin B in *Candida lusitaniae* infecting a human. *Antimicrob. Agents Chemother.* **16:**123–126.
106. **Park, S., R. Kelly, J. N. Kahn, J. Robles, M. J. Hsu, E. Register, W. Li, V. Vyas, H. Fan, G. Abruzzo, A. Flattery, C. Gill, G. Chrebet, S. A. Parent, M. Kurtz, H. Teppler, C. M. Douglas, and D. S. Perlin.** 2005. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical *Candida* sp. isolates. *Antimicrob. Agents Chemother.* **49:**3264–3273.
107. **Pasquale, T., J. R. Tomada, M. Ghannoun, J. Dipersio, and H. Bonilla.** 2008. Emergence of *Candida tropicalis* resistant to caspofungin. *J. Antimicrob. Chemother.* **61:**219.
108. **Pelletier, R., I. Alarie, R. Lagace, and T. J. Walsh.** 2005. Emergence of disseminated candidiasis caused by *Candida krusei* during treatment with caspofungin: case report and review of literature. *Med. Mycol.* **43:**559–564.
109. **Perlroth, J., B. Choi, and B. Spellberg.** 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med. Mycol.* **45:**321–346.
110. **Pfaller, M., and D. J. Diekema.** 2004. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida*. *Clin. Microbiol. Infect.* **10**(Suppl. 1)**:** 11–23.
111. **Pfaller, M. A., L. Boyken, R. J. Hollis, J. Kroeger, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2008. In vitro susceptibility of invasive isolates of *Candida* spp. to anidulafungin, caspofungin, and micafungin: six years of global surveillance. *J. Clin. Microbiol.* **46:**150–156.
112. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
113. **Pfaller, M. A., and D. J. Diekema.** 2004. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J. Clin. Microbiol.* **42:**4419–4431.
114. **Pfaller, M. A., D. J. Diekema, A. L. Colombo, C. Kibbler, K. P. Ng, D. L. Gibbs, V. A. Newell, and the Global Antifungal Surveillance Group.** 2006. *Candida rugosa*, an emerging fungal pathogen with resistance to azoles: geographic and temporal trends from the ARTEMIS DISK antifungal surveillance program. *J. Clin. Microbiol.* **44:**3578–3582.

115. **Pfaller, M. A., D. J. Diekema, M. Mendez, C. Kibbler, P. Erzsebet, S. C. Chang, D. L. Gibbs, V. A. Newell, and the Global Antifungal Surveillance Group.** 2006. *Candida guilliermondii*, an opportunistic fungal pathogen with decreased susceptibility to fluconazole: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program. *J. Clin. Microbiol.* **44:** 3551–3556.
116. **Pfaller, M. A., D. J. Diekema, M. G. Rinaldi, R. Barnes, B. Hu, A. V. Veselov, N. Tiraboschi, E. Nagy, D. L. Gibbs, and the Global Antifungal Surveillance Group.** 2005. Results from the ARTEMIS DISK global antifungal surveillance study: a 6.5-year analysis of susceptibilities of *Candida* and other yeast species to fluconazole and voriconazole by standardized disk diffusion testing. *J. Clin. Microbiol.* **43:**5848–5859.
117. **Pfaller, M. A., R. N. Jones, G. V. Doern, A. C. Fluit, J. Verhoef, H. S. Sader, S. A. Messer, A. Houston, S. Coffman, and R. J. Hollis.** 1999. International surveillance of blood stream infections due to *Candida* species in the European SENTRY program: species distribution and antifungal susceptibility including the investigational triazole and echinocandin agents. *Diagn. Microbiol. Infect. Dis.* **35:**19–25.
118. **Pfaller, M. A., R. N. Jones, G. V. Doern, H. S. Sader, S. A. Messer, A. Houston, S. Coffman, and R. J. Hollis.** 2000. Bloodstream infections due to *Candida* species: SENTRY antimicrobial surveillance program in North America and Latin America, 1997-1998. *Antimicrob. Agents Chemother.* **44:**747–751.
119. **Phan, Q. T., C. L. Myers, Y. Fu, D. C. Sheppard, M. R. Yeaman, W. H. Welch, A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol.* **5:**64.
120. **Pinjon, E., D. Sullivan, I. Salkin, D. Shanley, and D. Coleman.** 1998. Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. *J. Clin. Microbiol.* **36:**2093–2095.
121. **Rahman, D., M. Mistry, S. Thavaraj, S. J. Challacombe, and J. R. Naglik.** 2007. Murine model of concurrent oral and vaginal *Candida albicans* colonization to study epithelial host-pathogen interactions. *Microbes Infect.* **9:**615–622.
122. **Reichart, P. A., H. P. Philipsen, A. Schmidt-Westhausen, and L. P. Samaranayake.** 1995. Pseudomembranous oral candidiasis in HIV infection: ultrastructural findings. *J. Oral Pathol. Med.* **24:**276–281.
123. **Reuß, O., A. Vik, R. Kolter, and J. Morschhäuser.** 2004. The *SAT1* flipper, an optimized tool for gene disruption in *Candida albicans*. *Gene* **341:**119–127.
124. **Richardson, M., and C. Lass-Flörl.** 2008. Changing epidemiology of systemic fungal infections. *Clin. Microbiol. Infect.* **14**(Suppl. 4)**:**5–24.
125. **Roilides, E., E. Farmaki, J. Evdoridou, A. Francesconi, M. Kasai, J. Filioti, M. Tsivitanidou, D. Sofianou, G. Kremenopoulos, and T. J. Walsh.** 2003. *Candida tropicalis* in a neonatal intensive care unit: epidemiologic and molecular analysis of an outbreak of infection with an uncommon neonatal pathogen. *J. Clin. Microbiol.* **41:**735–741.
126. **Romeo, O., C. Racco, and G. Criseo.** 2006. Amplification of the hyphal wall protein 1 gene to distinguish *Candida albicans* from *Candida dubliniensis*. *J. Clin. Microbiol.* **44:**2590–2592.
127. **Roy, B., and S. A. Meyer.** 1998. Confirmation of the distinct genotype groups within the form species *Candida parapsilosis*. *J. Clin. Microbiol.* **36:**216–218.
128. **Rycovska, A., M. Valach, L. Tomaska, M. Bolotin-Fukuhara, and J. Nosek.** 2004. Linear versus circular mitochondrial genomes: intraspecies variability of mitochondrial genome architecture in *Candida parapsilosis*. *Microbiology* **150:**1571–1580.
129. **Sandven, P.** 2000. Epidemiology of candidemia. *Rev. Iberoam. Micol.* **17:**73–81.
130. **Sanglard, D., F. Ischer, and J. Bille.** 2001. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. *Antimicrob. Agents Chemother.* **45:**1174–1183.
131. **Sarvikivi, E., O. Lyytikainen, D. R. Soll, C. Pujol, M. A. Pfaller, M. Richardson, P. Koukila-Kahkola, P. Luukkainen, and H. Saxen.** 2005. Emergence of fluconazole resistance in a *Candida parapsilosis* strain that caused infections in a neonatal intensive care unit. *J. Clin. Microbiol.* **43:**2729–2735.
132. **Saville, S. P., A. L. Lazzell, C. Monteagudo, and J. L. Lopez-Ribot.** 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot. Cell* **2:**1053–1060.
133. **Seidenfeld, S. M., B. H. Cooper, J. W. Smith, J. P. Luby, and P. A. Mackowiak.** 1983. Amphotericin B tolerance: a characteristic of *Candida parapsilosis* not shared by other *Candida* species. *J. Infect. Dis.* **147:**116–119.
134. **Sharkey, L. L., W. L. Liao, A. K. Ghosh, and W. A. Fonzi.** 2005. Flanking direct repeats of *hisG* alter *URA3* marker expression at the *HWP1* locus of *Candida albicans*. *Microbiology* **151:**1061–1071.
135. **Shivaprakasha, S., K. Radhakrishnan, and P. M. Karim.** 2007. *Candida* spp. other than *Candida albicans*: a major cause of fungaemia in a tertiary care centre. *Indian J. Med. Microbiol.* **25:**405–407.
136. **Sipsas, N. V., R. E. Lewis, J. Tarrand, R. Hachem, K. V. Rolston, I. I. Raad, and D. P. Kontoyiannis.** 2009. Candidemia in patients with hematologic malignancies in the era of new antifungal agents (2001–2007): stable incidence but changing epidemiology of a still frequently lethal infection. *Cancer* **115:**4745–4752.
137. **Slutsky, B., J. Buffo, and D. R. Soll.** 1985. High-frequency switching of colony morphology in *Candida albicans*. *Science* **230:**666–669.
138. **Sobel, J. D.** 1998. Vulvovaginitis due to *Candida glabrata*. An emerging problem. *Mycoses* **41**(Suppl. 2)**:**18–22.
139. **Sobel, J. D.** 2007. Vulvovaginal candidosis. *Lancet* **369:**1961–1971.
140. **Soll, D. R.** 2009. Why does *Candida albicans* switch? *FEMS Yeast Res.* **9:**973–989.
141. **Song, J. W., J. H. Shin, D. H. Shin, S. I. Sung, D. Cho, S. J. Kee, M. G. Shin, S. P. Suh, and D. W. Ryang.** 2005. Differences in biofilm production by three genotypes of *Candida parapsilosis* from clinical sources. *Med. Mycol.* **43:**657–661.
142. **Spellberg, B. J., S. G. Filler, and J. E. Edwards, Jr.** 2006. Current treatment strategies for disseminated candidiasis. *Clin. Infect. Dis.* **42:**244–251.
143. **Spellberg, B. J., A. S. Ibrahim, V. Avanesian, Y. Fu, C. Myers, Q. T. Phan, S. G. Filler, M. R. Yeaman, and J. J. E. Edwards.** 2006. Efficacy of the anti-*Candida* rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J. Infect. Dis.* **194:**256–260.
144. **Srikantha, T., S. A. Lachke, and D. R. Soll.** 2003. Three mating type-like loci in *Candida glabrata*. *Eukaryot. Cell* **2:**328–340.
145. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase

substrate properties of *Candida albicans* Hwp1. *Science* **283:**1535–1538.
146. **Staab, J. F., and P. Sundstrom.** 2003. *URA3* as a selectable marker for disruption and virulence assessment of *Candida albicans* genes. *Trends Microbiol.* **11:**69–73.
147. **Staib, P., and J. Morschhaüser.** 1999. Chlamydospore formation on Staib agar as a species-specific characteristic of *Candida dubliniensis*. *Mycoses* **42:**521–524.
148. **Sterling, T. R., R. A. Gasser, Jr., and A. Ziegler.** 1996. Emergence of resistance to amphotericin B during therapy for *Candida glabrata* infection in an immunocompetent host. *Clin. Infect. Dis.* **23:**187–188.
149. **St-Germain, G., M. Laverdière, R. Pelletier, P. René, A. M. Bourgault, C. Lemieux, and M. Libman.** 2008. Epidemiology and antifungal susceptibility of bloodstream *Candida* isolates in Quebec: report on 453 cases between 2003 and 2005. *Can. J. Infect. Dis. Med. Microbiol.* **19:** 55–62.
150. **Stokes, C., G. P. Moran, M. J. Spiering, G. T. Cole, D. C. Coleman, and D. J. Sullivan.** 2007. Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*. *Fungal Genet. Biol.* **44:**920–931.
151. **Suh, S. O., N. H. Nguyen, and M. Blackwell.** 2008. Yeasts isolated from plant-associated beetles and other insects: seven novel *Candida* species near *Candida albicans*. *FEMS Yeast Res.* **8:**88–102.
152. **Sullivan, D., and D. Coleman.** 1997. *Candida dubliniensis*: an emerging opportunistic pathogen. *Curr. Top. Med. Mycol.* **8:**15–25.
153. **Sullivan, D., and D. Coleman.** 1998. *Candida dubliniensis*: characteristics and identification. *J. Clin. Microbiol.* **36:**329–334.
154. **Sullivan, D., K. Haynes, J. Bille, P. Boerlin, L. Rodero, S. Lloyd, M. Henman, and D. Coleman.** 1997. Widespread geographic distribution of oral *Candida dubliniensis* strains in human immunodeficiency virus-infected individuals. *J. Clin. Microbiol.* **35:**960–964.
155. **Sullivan, D. J., and D. C. Coleman.** 2002. Molecular approaches to identification and typing of *Candida* species, p. 427–441. *In* R. A. Caldeone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
156. **Sullivan, D. J., G. P. Moran, and D. C. Coleman.** 2005. *Candida dubliniensis*: ten years on. *FEMS Microbiol. Lett.* **253:**9–17.
157. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141:**1507–1521.
158. **Tan, T. Y., A. L. Tan, N. W. Tee, L. S. Ng, and C. W. Chee.** 2010. The increased role of non-*albicans* species in candidaemia: results from a 3-year surveillance study. *Mycoses* **53:**515–521.
159. **Tavanti, A., A. D. Davidson, N. A. R. Gow, M. C. J. Maiden, and F. C. Odds.** 2005. *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. *J. Clin. Microbiol.* **43:**284–292.
160. **Tavanti, A., L. A. Hensgens, E. Ghelardi, M. Campa, and S. Senesi.** 2007. Genotyping of *Candida orthopsilosis* clinical isolates by amplification fragment length polymorphism reveals genetic diversity among independent isolates and strain maintenance within patients. *J. Clin. Microbiol.* **45:**1455–1462.
161. **Tortorano, A. M., J. Peman, H. Bernhardt, L. Klingspor, C. C. Kibbler, O. Faure, E. Biraghi, E. Canton, K. Zimmermann, S. Seaton, and R. Grillot.** 2004. Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study. *Eur. J. Clin. Microbiol. Infect. Dis.* **23:**317–322.
162. **Trofa, D., A. Gácser, and J. D. Nosanchuk.** 2008. *Candida parapsilosis*, an emerging fungal pathogen. *Clin. Microbiol. Rev.* **21:**606–625.
163. **Tsuchimori, N., L. L. Sharkey, W. A. Fonzi, S. W. French, J. E. Edwards, and S. G. Filler.** 2000. Reduced virulence of *HWP1*-deficient mutants of *Candida albicans* and their interactions with host cells. *Infect. Immun.* **68:**1997–2002.
164. **Tumbarello, M., G. Caldarola, E. Tacconelli, G. Morace, B. Posteraro, R. Cauda, and L. Ortona.** 1996. Analysis of risk factors associated with the emergence of azole resistant oral candidosis in the course of HIV infection. *J. Antimicrob. Chemother.* **38:**691–699.
165. **van Asbeck, E., K. V. Clemons, M. Martinez, A. J. Tong, and D. A. Stevens.** 2008. Significant differences in drug susceptibility among species in the *Candida parapsilosis* group. *Diagn. Microbiol. Infect. Dis.* **62:**106–109.
166. **van Asbeck, E. C., K. V. Clemons, and D. A. Stevens.** 2009. *Candida parapsilosis*: a review of its epidemiology, pathogenesis, clinical aspects, typing and antimicrobial susceptibility. *Crit. Rev. Microbiol.* **35:**283–309.
167. **Vanden Bossche, H., P. Marichal, F. C. Odds, L. Le Jeune, and M.-C. Coene.** 1992. Characterization of an azole-resistant *Candida glabrata* isolate. *Antimicrob. Agents Chemother.* **36:**2602–2610.
168. **Vandeputte, P., G. Larcher, T. Berges, G. Renier, D. Chabasse, and J. P. Bouchara.** 2005. Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. *Antimicrob. Agents Chemother.* **49:**4608–4615.
169. **Vandeputte, P., G. Tronchin, T. Bergès, C. Hennequin, D. Chabasse, and J. P. Bouchara.** 2007. Reduced susceptibility to polyenes associated with a missense mutation in the *ERG6* gene in a clinical isolate of *Candida glabrata* with pseudohyphal growth. *Antimicrob. Agents Chemother.* **51:**982–990.
170. **Vandeputte, P., G. Tronchin, G. Larcher, E. Ernoult, T. Bergès, D. Chabasse, and J. P. Bouchara.** 2008. A nonsense mutation in the *ERG6* gene leads to reduced susceptibility to polyenes in a clinical isolate of *Candida glabrata*. *Antimicrob. Agents Chemother.* **52:**3701–3709.
171. **Vargas, K. G., and S. Joly.** 2002. Carriage frequency, intensity of carriage, and strains of oral yeast species vary in the progression to oral candidiasis in human immunodeficiency virus-positive individuals. *J. Clin. Microbiol.* **40:**341–350.
172. **Verma, A. K., K. N. Prasad, M. Singh, A. K. Dixit, and A. Ayyagari.** 2003. Candidaemia in patients of a tertiary health care hospital from north India. *Indian J. Med. Res.* **117:**122–128.
173. **Viscoli, C., C. Girmenia, A. Marinus, L. Collette, P. Martino, B. Vandercam, C. Doyen, B. Lebeau, D. Spence, V. Krcmery, F. De Pauw, and F. Meunier.** 1999. Candidemia in cancer patients: a prospective, multicentre surveillance study by the Invasive Fungal Infection Group (IFIG) of the European Organization for Research and Treatment of Cancer (EORTC). *Clin. Infect. Dis.* **28:**1071–1079.
174. **Viudes, A., J. Pemán, E. Cantón, M. Salavert, P. Ubeda, J. L. López-Ribot, and M. Gobernado.** 2002. Two cases of fungemia due to *Candida lusitaniae* and a literature review. *Eur. J. Clin. Microbiol. Infect. Dis.* **21:**294–299.
175. **Voss, E. G.** 1983. *International Code of Botanical Nomenclature*. Dr. W. Junk Publishers, The Hague, The Netherlands.

176. **Walsh, T. J., and W. G. Merz.** 1986. Pathologic features in the human alimentary tract associated with invasiveness of *Candida tropicalis*. *Am. J. Clin. Pathol.* **85:**498–502.
177. **Wang, J. L., S. C. Chang, P. R. Hsueh, and Y. C. Chen.** 2004. Species distribution and fluconazole susceptibility of *Candida* clinical isolates in a medical center in 2002. *Microbiol. Immunol. Infect.* **37:**236–241.
178. **Wilson, L. S., C. M. Reyes, M. Stolpman, J. Speckman, K. Allen, and J. Beney.** 2002. The direct cost and incidence of systemic fungal infections. *Value Health* **5:**26–34.
179. **Wingard, J. R.** 1995. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. *Clin. Infect. Dis.* **20:**115–125.
180. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317.
181. **Wong, S., M. A. Fares, W. Zimmermann, G. Butler, and K. H. Wolfe.** 2003. Evidence from comparative genomics for a complete sexual cycle in the 'asexual' pathogenic yeast *Candida glabrata*. *Genome Biol.* **4:**R10.
182. **Wu, W., C. Pujol, S. R. Lockhart, and D. R. Soll.** 2005. Chromosome loss followed by duplication is the major mechanism of spontaneous mating-type locus homozygosis in *Candida albicans*. *Genetics* **169:**1311–1327.
183. **Yarrow, D., and S. A. Meyer.** 1978. Proposal for amendment of the diagnosis of the genus *Candida* Berkhout nom. cons. *Int. J. Syst. Biol.* **28:**611–615.
184. **Zaoutis, T. E., J. Argon, J. Chu, J. A. Berlin, T. J. Walsh, and C. Feudtner.** 2005. The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. *Clin. Infect. Dis.* **41:**1232–1239.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch3

3

Comparative Genomics of *Candida* Species

GERALDINE BUTLER

Changes in technology since the last edition of Candida *and Candidiasis* are probably more apparent in the field of genomics than in any other area. At that time (2002), the genome sequence of one isolate of *Candida albicans* (SC5314) was almost complete, and the data were available to the public. The only other fungal genome sequences available were those of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (54, 162). A diploid assembly of *C. albicans* (assembly 19) was published in 2004 (77), an improved annotation became available in 2005 (9), and a haploid assembly (assembly 21) was reported in 2007 (155). Today, more than 80 fungal genomes have been reported (158).

Whereas *C. albicans* is one of the most common causes of disease, other *Candida* species are also major causes of infection. These include relatives of *C. albicans* such as *C. tropicalis* and *C. parapsilosis*, as well as the more distantly related species *Candida glabrata* (Fig. 1). Together these species make up the "big four" and are responsible for approximately 90% of *Candida* infections (116). Other *Candida* species are more rarely associated with disease, such as *Candida dubliniensis* (<0.1%), which predominantly infects human immunodeficiency virus-infected patients, and *Candida lusitaniae* and *Candida guilliermondii*, which are responsible for ~1.5% of infections in the United States (116). *Candida krusei* (2 to 4% of global infections) is common among patients with hematologic malignancies (160).

The term *Candida* is not a useful indication of phylogenetic relationship, as it refers to the lack of an observed sexual cycle. For some species, subsequent discovery of a sexual cycle led to a new designation. For example, the sexual forms (teleomorphs) of *C. lusitaniae*, *C. guilliermondii*, and *C. famata* are designated *Clavispora lusitaniae*, *Pichia guilliermondii*, and *Debaryomyces hansenii*, respectively. However, apart from *C. glabrata* and *C. krusei*, the major disease-causing *Candida* species belong to the same clade (Fig. 1). This is referred to as the CTG clade, as in these species CTG encodes serine rather than leucine (113, 128). Evolution of the CTG codon is discussed in chapter 4. The clade also includes *Lodderomyces elongisporus*, which is sometimes isolated from bloodstream infections and sometimes misidentified as its close relative *C. parapsilosis* (88). Other clade members are rarely, if ever, associated with disease. These include *D. hansenii*, a salt-tolerant yeast associated with cheese, and *Pichia stipitis*, a xylose-degrading yeast that is related to species found as endosymbionts of passalid beetles that inhabit rotting wood (144).

Some major international efforts in the last few years have led to the sequencing of 10 genomes from the CTG clade (Table 1). The sequence of *D. hansenii* was reported in 2004 by the Génolevures consortium (33), and that of *P. stiptis* in 2007 (75). In 2009, the genomes of a second isolate of *C. albicans* (WO-1) and single isolates of *C. tropicalis*, *C. parapsilosis*, *L. elongisporus*, *C. guilliermondii*, and *C. lusitaniae* were completed (12), followed closely by that of *C. dubliniensis* (73). The genomes of the CTG clade species vary in size from 10.6 Mb (*C. guilliermondii*) to 15.4 Mb (*P. stipitis* and *L. elongisporus*). However, the total gene numbers are similar (Table 1). The vast array of genome information available enables detailed comparisons, such as between pathogenic and nonpathogenic species and between common and rare pathogens. Although the sequence of *C. glabrata* was completed in 2004 (33), its phylogenetic position outside the CTG clade makes it difficult to include in comparative analysis. This chapter therefore focuses on the *Candida* species within the CTG clade. For other information related to this topic, see chapter 4.

SINGLE NUCLEOTIDE POLYMORPHISMS

Several of the species (*C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, and *L. elongisporus*) have diploid genomes, so it is possible to compare the single nucleotide polymorphisms (SNPs) between the homologous chromosomes. The frequency of SNPs varies from 1 per 220 bases in *L. elongisporus* to 1 per 15,553 bases in *C. parapsilosis* (12). The distribution is relatively uniform in *C. parapsilosis* but varies substantially in the others. In *C. dubliniensis*, for example, the overall number of SNPs is significantly lower than in *C. albicans*, and the frequency ranges from 1 per 635 bases on chromosome 6 to 1 per 12,555 bases on chromosome 1 (73). Chromosome 1 is almost homozygous, as the small numbers of SNPs present are clustered in one region

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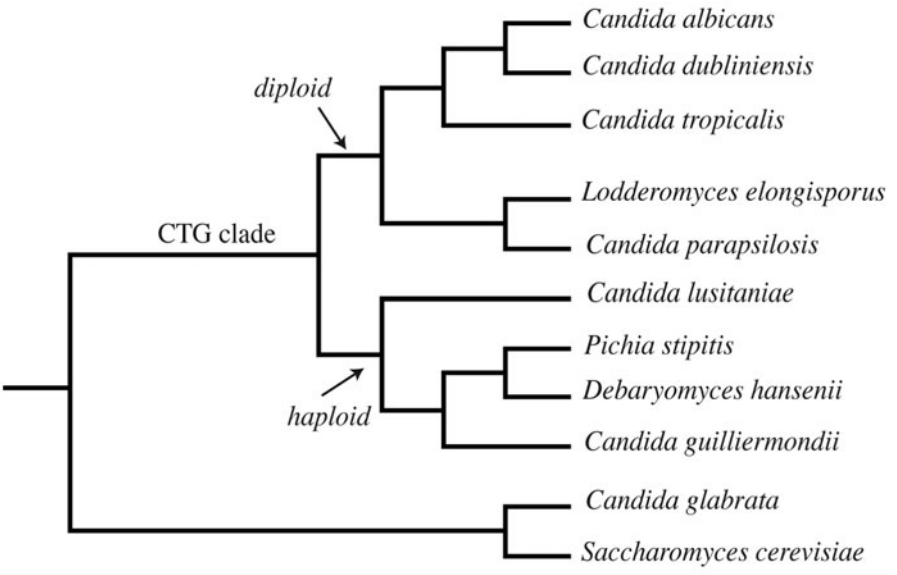

FIGURE 1 Phylogenetic relationship of the sequenced *Candida* species. The tree was constructed using a supertree approach, including all 10 genomes from the CTG clade and *C. glabrata* and *S. cerevisiae*. Methods are described in the work of Fitzpatrick et al. (41). The subclades that contain haploid and diploid genomes are indicated. 10.1128/9781555817176.ch3f1

towards the center. There are very large homozygous tracts on all the other chromosomes, and chromosomes 3, 7, and R are mostly homozygous. The genomes of other diploid species also contain smaller homozygous tracts, usually extending to the ends of the chromosomes. The *L. elongisporus* genome is shown in Fig. 2.

Interestingly, the homozygous tracts differ between the two *C. albicans* isolates (SC5314 and WO-1), and the level of homozygosity is higher overall in WO-1 (approximately 30% of the genome) (12). Loss of heterozygosity (LOH) at defined regions in *C. albicans* has been shown to occur during passaging through a mouse model, most likely associated with gene conversion and mitotic recombination (44, 45). LOH can have dramatic phenotypic effects, such as the acquisition of drug resistance (22, 23, 34). Long-range LOH events have also been described, such as between pairs of *C. albicans* isolates in healthy human carriers, which may be caused by break-induced replication (BIR) (30). In several instances, Diogo et al. (30) showed that tRNA genes are located near several of the LOH boundaries. In *S. cerevisiae*, gross chromosomal rearrangements are often associated with tRNA genes (21, 31, 35, 165). BIR is induced by double-stranded breaks, which occur spontaneously in replication slow zones (RSZ) in *S. cerevisiae* (16). Because RSZ have been mapped to tRNAs (28), Diogo et al. (30) suggest that the long regions of LOH in *C. albicans* are formed by BIR initiated at the tRNA genes. The presence of long LOH tracts in all the diploid *Candida* species (12) indicates that BIR plays a major role in the evolution of *Candida* genomes. However, any association with tRNA genes and the phenotypic consequences in species other than *C. albicans* remains to be investigated.

TABLE 1 Sequenced genomes in the CTG clade

Strain	Genome size (Mb)	No. of genes	Ploidy	Pathogenesis	Mating	ORF identifier[a]	Genome reference
C. albicans SC5314	14.3	6,107	Diploid	+++	Parasexual	orf19	Assembly 21, CGD
C. albicans WO-1	14.4	6,159	Diploid	+++	Parasexual	CAWG	12
C. dubliniensis CD36	14.6	5,758	Diploid	+	Parasexual	Cd36	73
C. tropicalis MYA-3404	14.5	6,528	Diploid	++	?	CTRG	12
C. parapsilosis CDC 317	13.1	5,733	Diploid	++	?	CPAG, cpar	12
L. elongisporus NRRL YB-4239	15.4	5,802	Diploid	+/–	?	LELG	12
C. guilliermondii ATCC 6260	10.6	5,920	Haploid	+	Heterothallic	PGUG	12
C. lusitaniae ATCC 42720	12.1	5,941	Haploid	+	Heterothallic	CLUG	12
D. hansenii CBS 767	12.2	6,906	Haploid	–	Homothallic	DEHA	33
P. stipitis CBS 6054	15.4	5,841	Haploid	–	Homothallic	PICST	75

[a]ORF identifiers assigned by the genome sequencing centers. Two ORF naming systems have been used with *C. parapsilosis*: CPAG (from the Broad Institute, http://www.broadinstitute.org/annotation/genome/candida_group/MultiHome.html) and cpar (from the Butler group, http://cgob.ucd.ie).

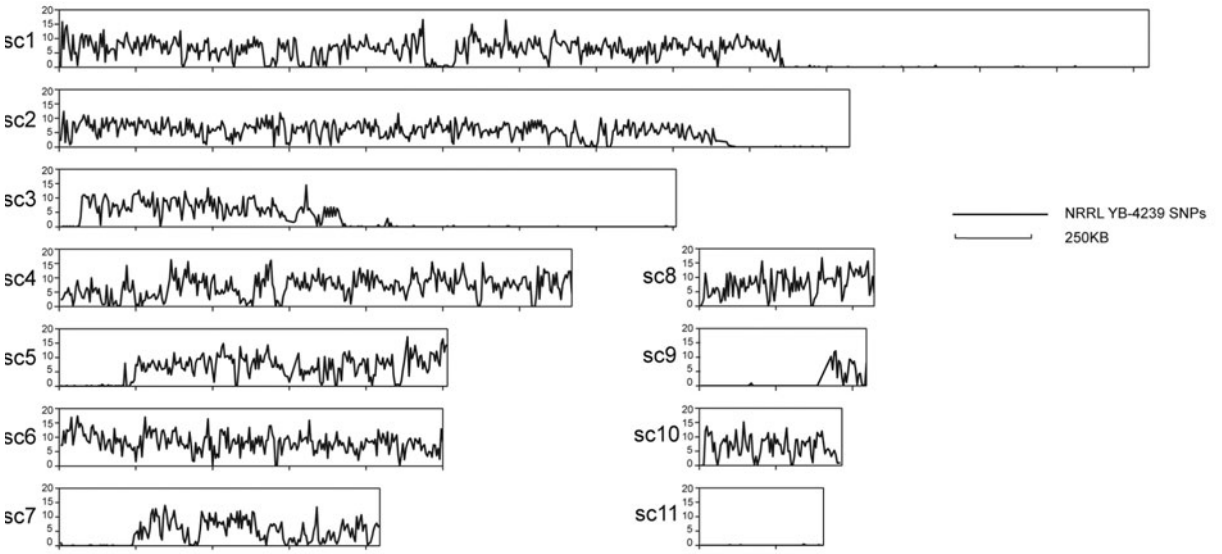

FIGURE 2 Distribution of SNPs in *L. elongisporus*. SNP frequency (SNPs per kilobase, normalized by the fraction of high-quality aligned positions) for 10-kb windows is plotted along *L. elongisporus* scaffolds. sc, supercontig, approximately corresponding to chromosomes. Reproduced from reference 12 with permission. 10.1128/9781555817176.ch3f2

REPETITIVE DNA ELEMENTS

Major Repeat Sequence

Almost half the isolates of C. *albicans* have a "standard" karyotype, and the remainder exhibit rearrangements and translocations predominantly at the major repeat sequence (MRS) (17, 94). There is an MRS on every chromosome except chromosome 3, and some have two. The MRS consists of a repetitive sequence (RPS) repeated up to 50 times, sandwiched between two unique elements called HOK and RB2 that occur once per MRS (18, 19). The RPS repeat unit is just over 2 kb and contains smaller repeats of 172 bp called alts (72). The alt sequences vary between repeats, but they also contain a conserved sequence that includes a recognition site for the SfiI restriction enzyme site. SfiI maps are therefore very useful for karyotype analysis (20).

C. *dubliniensis* chromosomes also contain MRSs with a conserved RPS element (76). However, unlike in C. *albicans*, chromosome R in C. *dubliniensis* has no MRS, and chromosome 3 does (93). In addition, the C. *dubliniensis* MRSs have a larger number of RPSs, which may partly explain the large variation in karyotype between isolates (76). Potential MRS-like sequences were identified in C. *tropicalis*, but they are not present in any of the other *Candida* genomes (12).

Telomeres

Telomeres from C. *albicans*, C. *tropicalis*, and C. *guilliermondii* and several other yeasts were initially identified by cloning (99, 100). The C. *albicans* telomeres consist of a 23-bp repeat and are identical from several isolates (100, 127). The repeat unit is substantially bigger than that found in *S. cerevisiae* but is similar in size to that of the other *Candida* species (Table 2) (99). Some C. *tropicalis* isolates are reported to have two types of repeat, which differ by a single base (99). The assemblies of many of the sequenced *Candida* genomes did not include the telomeres. However, candidate repeats were identified from unassembled reads and then linked to scaffold ends by using paired end sequences (12). This confirmed some of the earlier findings and identified novel telomeres in *L. elongisporus* and C. *lusitaniae*. The orientation was established and novel telomeres were isolated from C. *metapsilosis*, C. *orthopsilosis*, and C. *sojae* by PCR amplification (59).

Transcription of the telomeres produces RNA molecules that range from 800 (C. *guilliermondii*) to 1,800 (C. *parapsilosis*) nucleotides (59). Although the RNA sequences vary among species, they also contain conserved regions, including a consensus binding site for the reverse transcriptase protein (59). Processing of the 3′ end appears to involve intron splicing, similar to the mechanism observed in *S. pombe* and different from that in *S. cerevisiae* (8, 59). Analysis of the *Candida* RNA sequences suggests that they are folded into pseudoknots containing U-A-U triples, a structure that is required for telomerase function in yeasts, ciliates, and vertebrates (59, 119, 134, 152). Orthologs of Est2, the catalytic subunit of telomerase, are present in all the *Candida* species, with the possible exception of *P. stipitis* (59). However, Est1, which is an allosteric activator of catalytic activity, is missing from C. *parapsilosis* and *L. elongisporus*, and its function may be replaced by a different protein in these species (59).

Centromeres

The centromeres in *S. cerevisiae* and closely related species (including C. *glabrata*) are defined by short (<200-bp) sequences containing two conserved regions or centromere DNA elements, CDEI (8 bp) and CDEIII (125 bp), separated by an AT-rich region (CDEII). A single nucleosome containing Cse4 (histone H3-like protein) binds to the centromere (63, 83). These are known as "point" centromeres and are very easy to identify in multiple chromosomes in the same species, or even between species. In contrast, C. *albicans* and C. *dubliniensis* have regional centromeres (114, 129). They are defined by binding of several Cse4 (histone H3 variant) nucleosomes, are relatively large (3 to 5 kb), and share no obvious sequence motifs (103, 129). If the

TABLE 2 Telomere sequences in *Candida* species

Species	Telomere motif	Reference(s)
C. albicans	TGTACGGATGTCTAACTTCTTGG	100
C. dubliniensis	TGTACGGATGTCTAACTTCTGG	59, 73
C. tropicalis	GTGTAMGGATGTCACGATCATTG	12, 99
C. parapsilosis	GGTCCGGATGTTGATTATACTGA	59
C. orthopsilosis	GGTTAGGATGTAGACAATACTGC	59
C. metapsilosis	GGTTAGGATGTCCAAAGTATTGA	59
L. elongisporus	CGGTGTAAGGATGCACTTGAAACT	12, 59
C. guilliermondii	GTGTACTGGTGT	99
C. lusitaniae	TCTTTAGGGAGGTACTGATGT	12, 59
D. hansenii	GGATGTTGAGGTGTAG	87
P. stipitis	GGATCTTTTCACGTCTTGCGGTAT	75

endogenous centromere is removed, neocentromeres form in regions of low gene density with flanking repeated DNA sequences (82). The location of the centromeres (though not the sequence) is conserved between *C. albicans* and *C. dubliniensis*, and there are some shared repeated sequences in the regions outside the Cse4-binding sites (114). However, the lack of synteny makes it difficult to predict the position of centromeres in the other *Candida* species.

It has been proposed that regional centromeres were present in the ancestor of the Saccharomycotina and that the point centromeres in the *Saccharomyces* lineage were formed when a 2μm-like plasmid integrated into the chromosomes, displacing their previous regional centromeres (95).

Retrotransposons

Families of long terminal repeat (LTR) and non-LTR retrotransposons have been well characterized in *C. albicans* (55–58). *C. albicans* contains more retrotransposon families than *S. cerevisiae*; however, most are nonfunctional or are present in low copy numbers (57). Members of the Ty1/Copia class are intact and are found in the genomes of many *Candida* species (12, 75). In *P. stipitis*, several copies of Tps5 (Ty5-like) retrotransposon are found clustered in a single location on each chromosome (75).

Rates of transmission of transposable elements are predicted to be high in species with sexual reproduction and low (and possibly zero) in asexual populations (163). There is little obvious difference in the number of transposable elements in fully sexual *Candida* species (such as *C. guilliermondii*, *C. lusitaniae*, and *D. hansenii*) and asexual ones (such as *C. tropicalis* and *C. parapsilosis*). The families of retrotransposons present vary among species, but all species analyzed contain some (12). However, an in-depth analysis of all sequenced *Candida* species has yet to be carried out. In addition, asexual species are likely to be derived from sexual ones and therefore may have inherited much of their retrotransposon content. It is likely that the transposable elements would gradually deteriorate in asexual species, and it is notable that the genomes of both *C. albicans* and *C. dubliniensis* contain several partial or nonfunctional elements (57, 73).

COMPARISON OF TWO *C. ALBICANS* GENOMES

C. albicans SC5314 was originally chosen for sequencing, as it is the isolate most commonly used in molecular analysis (77). The second isolate sequenced in 2009 (12), *C. albicans* WO-1, belongs to a different population subgroup (146). It is homozygous at the mating type-like locus (*MTL*), and it was the isolate used to characterize the role of white-opaque phenotype switching in mating (7, 89, 102, 138). Analysis of the two *C. albicans* genome sequences (together with those of the other *Candida* clade species) led to the identification of 74 new genes in *C. albicans*, the extension of 10 open reading frames (ORFs), and the removal of >100 dubious genes (12).

The genomes of *C. albicans* SC5314 and WO-1 are largely colinear, although there has been recombination at the MRS site on chromosome 5 (20). There are also 12 inversions, ranging in size from 5 to 94 kb (12). One inversion on chromosome R surrounds a cluster of biotin biosynthetic genes and appears to have resulted from a recombination between two members of the oligopeptide transporter (OPT) gene family (42). Another member of the OPT family (*orf19.3749*) is apparently missing from *C. albicans* WO-1.

Several putative differences between SC5314 and WO-1 may be caused by annotation errors. For example, a region containing several genes (from *CAWG_03408* to *CAWG_03415*) appears to be unique to WO-1 (12). However, orthologous ORFs were present in early assemblies of SC5314 and removed from later ones. Several of the predicted ORFs are very small, and perhaps some of the adjacent ones should be merged to give longer ORFs. For example, one of the removed genes (*orf19.1025*) is very similar to the C terminus of *orf19.3726*. It is therefore possible that these ORFs are not "dubious" and should be reinstated in the SC5314 annotation.

Other regions of incongruence between the genomes include the mating type-like loci and subtelomeric regions.

ANALYSIS OF GENE FAMILIES

Even before the first *C. albicans* genome sequence was released, several gene families with roles in pathogenesis had been identified. These include agglutinin-like sequence (ALS) adhesins (64), secreted aspartyl proteases (108), secreted lipases, iron transporters, and ferric reductases. The first whole-genome analysis identified additional families of OPTs, putative estrogen-binding proteins, sphingomyelinases, and cytochrome P-450 proteins (77). Braun et al. (9) subsequently identified >400 multigene families, including drug transporters and amino acid permeases.

Butler et al. (12) exploited the availability of sequences from several species to carry out a phylogenomic analysis of multigene families. Gene families were identified from seven CTG clade species (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *L. elongisporus*, *D. hansenii*, *C. lusitaniae*, and *C. guilliermondii*) and nine members of the *Saccharomyces* clade (including *C. glabrata*). Gene family enrichment and depletion in all of the pathogens (*C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. lusitaniae*, *C. guilliermondii*, and *C. glabrata*) or in the common pathogens only (*C. albicans*, *C. tropicalis*, and *C. parapsilosis*) were determined relative to the other species. Twenty-one families are enriched in the pathogenic species, five of which are particularly enriched in the strong pathogens (Table 3). These include three families of cell wall genes: glycosylphosphatidylinositol (GPI) family 17 (ALS-like adhesins), GPI family 13 (Pga30-like), and GPI family 18 (Hyr/Iff-like) (see chapter 14). The remaining two are a leucine-rich repeat family and an unclassified family.

ALS Family

ALS genes encode adhesins, which are important for cell-cell adhesion and for adherence of *Candida* cells to other surfaces (64, 65). The first *ALS* gene was identified in *C. albicans* in 1995, from a screen for genes with induced expression during hyphal growth (66). Southern blot analysis indicated that *ALS1* was part of a larger gene family, finally shown to consist of eight members (65). *ALS* genes consist of three domains: a 5′ region, which encodes a secretion signal and 300 to 330 amino acids with relatively little glycosylation, followed by approximately 100 amino acids rich in serine and threonine that is likely to be heavily glycosylated; a central domain encoding large numbers of a tandemly repeated sequence; and a 3′ domain which is poorly conserved but encodes a Ser/Thr-rich region with many N glycosylation sites and a GPI anchor (64, 65). The general structure is conserved with other yeast adhesins such as agglutinins and flocculins in *S. cerevisiae* and the *EPA* proteins of *C. glabrata*, although the sequences are very different in each species (32). Als proteins appear to be localized at the cell surface (25).

Similarities between Als1 and alpha-agglutinin from *S. cerevisiae* were the first indication that the *ALS* family may be involved in adhesion (66). This was supported by the demonstration that heterologous expression of *ALS1* and *ALS5* in *S. cerevisiae* confers the ability to bind to epithelial cells and other cell components (47, 50). Als1 was also subsequently shown to be required for flocculation and adherence of *C. albicans*, though deletions do not have reduced adherence to all cell types (46, 167). *ALS2* and *ALS4* probably play minor roles in adhesion (169). Deletion of *ALS5*, *ALS6*, or *ALS7* reduces adhesion to human cells (168).

ALS3 is likely to play the most important role in adhesion. Gene deletions cause a large reduction in adherence to endothelial and epithelial cells (167). *ALS3* and *ALS1* are important for adherence to plastic surfaces and for biofilm development. Biofilms generated by *als3* deletions are defective in vitro, though not in vivo (110, 166). However, Nobile et al. (111) have shown that Als1 and Als3 have redundant functions in biofilm development and that they act in conjunction with a different adhesin, Hwp1. Hwp1 was originally assumed to be localized to conjugation tubes of *MTL***a**/**a** but not α/α cells (24), leading to the suggestion that it may share a common ancestry with **a**-agglutinins, and Als proteins with alpha-agglutinins (111, 139). However, it was subsequently shown that Hwp1 is found on the surface of cells of both mating types (36).

Als3 is also important for processes other than adhesion. It behaves as an invasin by binding to clathrin, which is required for endocytosis and internalization of *C. albicans* cells (106, 117). Finally, *ALS3* is required for growth on ferritin as a sole iron source and for the uptake of iron from ferritin by hyphal cells (3).

The precise number of *ALS* genes in the other *Candida* species is likely to be overestimated, caused by difficulties in assembling the repeated regions (65). However, there are large numbers in the genomes of the common pathogenic species *C. tropicalis* (up to 16) and *C. parapsilosis* (approximately 5) (12). The nonpathogenic species *L. elongisporus* has four *ALS*-like genes. The role of the family in adhesion and pathogenesis of species other than *C. albicans* is not clear. In *C. parapsilosis*, for example, there is no dramatic change in expression of *ALS* family genes in biofilms (125). Expression of one *ALS* family member (*CPAG_05054*) is induced approximately threefold after 24 h of biofilm growth, and expression of another (*CPAG_05056*) is slightly increased in 50-h biofilms. In *C. albicans*, expression of *ALS1* is upregulated in biofilms, and there is some evidence that expression of *ALS3* is reduced (49, 109). However, expression of the *C. parapsilosis ALS* genes has not been investigated in different biofilm models, and it is possible that different *ALS* alleles may play different roles.

It is extremely difficult to determine the ancestry of the Als proteins, both because of the presence of internal repeats and because of the likelihood of recombination or gene conversion between copies in the individual species.

TABLE 3 Gene families enriched in pathogenic *Candida* species[a]

Rank	Gene family[b]	Description
1	**CF8711**	**GPI family 18 (Hyr/Iff-like)**
2	**CF7734**	**Leucine-rich repeat (IFA/FGR38-like)**
3	CF10326	Ferric reductase family
4	CF10581	Reductase family
5	**CF1318**	**GPI family 17 (ALS-like adhesins)**
6	**CF9063**	**GPI family 13 (Pga30-like)**
7	**CF739**	**Unclassified**
8	CF10133	Cell wall mannoprotein biosynthesis
9	CF11051	Major facilitator transporters
10	CF10555	OPTs
11	CF4945	Unclassified
12	CF10190	Amino acid permeases
13	CF5137	Sphingomyelin phosphodiesterases
14	CF7425	FGR6 family (filamentous growth)
15	CF3922	Secreted lipases
16	CF10446	Cytochrome P-450 family
17	CF11017	Amino acid permeases
18	CF8543	Zinc finger transcription factors
19	CF7011	Unclassified
20	CF8942	Predicted transmembrane family
21	CF8593	Unclassified secreted family

[a]Adapted from reference 12.

[b]*Candida* gene families are available at http://www.broadinstitute.org/annotation/genome/candida_group/MultiHome.html. The families in bold are particularly enriched in the strong pathogens (*C. albicans*, *C. tropicalis*, *and C. parapsilosis*).

However, Jackson et al. (73) carried out a detailed comparison of *ALS* genes between C. *albicans* (8 members) and the closely related species C. *dubliniensis* (6 members) and showed by analysis of phylogeny and gene order that most of the family (*ALS1*, *ALS2*, *ALS4*, *ALS6*, *ALS7*, and *ALS9*) are orthologous (Fig. 3). *ALS5* is specific to C. *albicans* and probably arose from a recent duplication of *ALS1*. Similarly, Cd36_64800 most likely arose in C. *dubliniensis* as a duplicate of Cd36_65010 (*ALS2*). Species-specific amplifications have also occurred in the other *Candida* species (12). Most interestingly, however, *ALS3* is specific to C. *albicans*, and a positional ortholog is not present in C. *dubliniensis*, C. *tropicalis*, or any other *Candida* species. This suggests that the particular properties of Als3 (invading tissues and acquisition of iron) are likely to be specific to C. *albicans* and may at least partly explain why this species is such a successful pathogen.

Hyr/Iff Family

The Hyr/Iff gene family also encodes proteins associated with cell wall assembly. The first member of the family (*HYR1*) was identified from C. *albicans* in 1996, in a screen for genes that are expressed in hyphae (4). There are at least 11 members in C. *albicans*, which is expanded to 17 in C. *parapsilosis* and 18 in C. *tropicalis* (12). Other *Candida* species also contain significant numbers of copies (e.g., 7 in C. *lusitaniae* and 3 in C. *guilliermondii*). The structure of Hyr/Iff proteins is similar to that of Als and other families of cell wall proteins; they contain an N-terminal secretion signal, intragenic tandem repeats, and a GPI anchor, and they

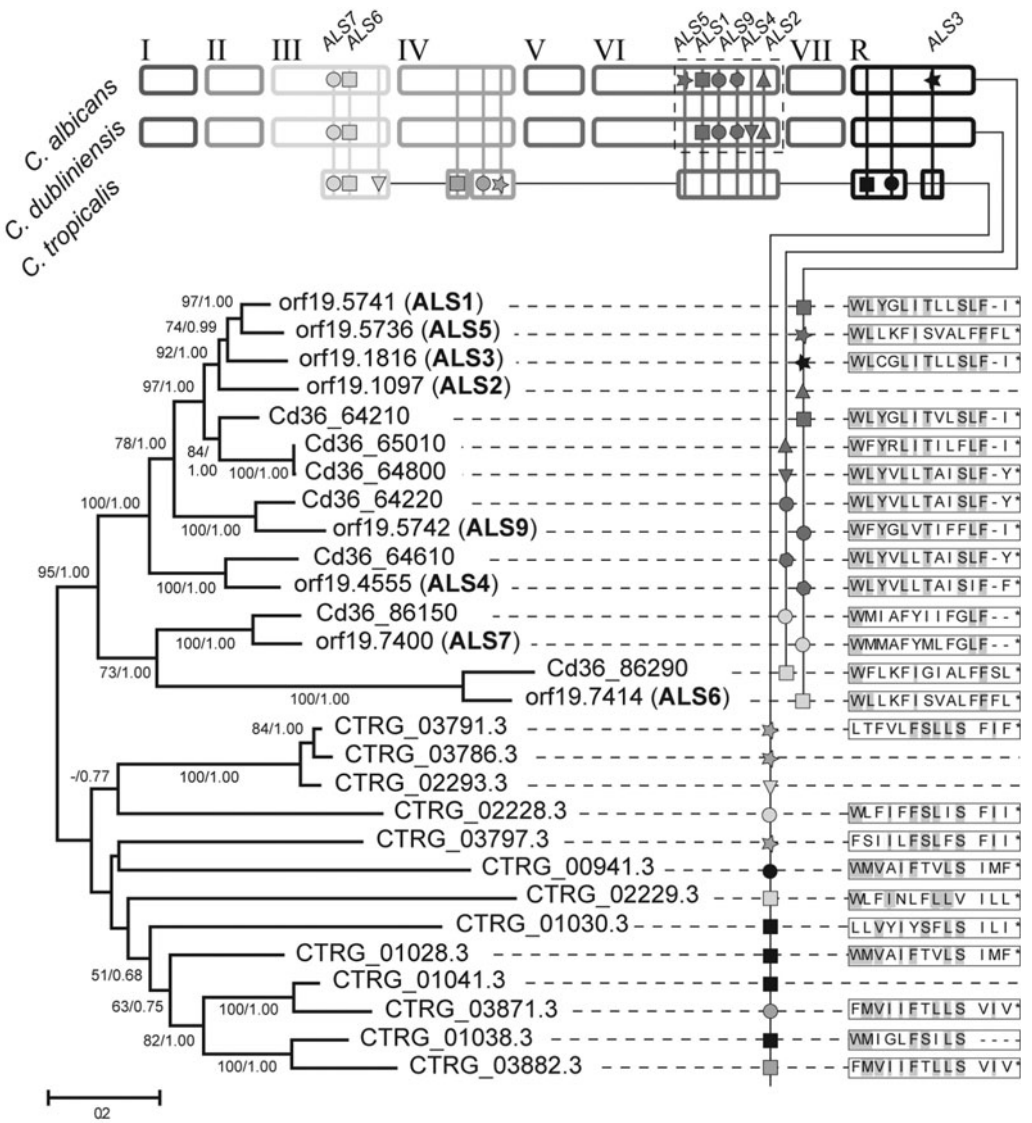

FIGURE 3 Comparative analysis of *ALS* sequences from *Candida* species. (Top panel) Genomic distribution of *ALS* genes in C. *albicans*, C. *dubliniensis*, and C. *tropicalis*. (Bottom panel) Maximum-likelihood phylogeny of *ALS* genes from the three species shown in the top panel. Terminal nodes are labeled with the same symbols and gene names as shown in the top panel. A conserved 14-amino-acid sequence at the C terminus is shown. *ALS3* is present only in C. *albicans*. Reproduced from *Genome Research* (73) with permission from the publisher. 10.1128/9781555817176.ch3f3

are heavily glycosylated. Expansion and variation in the repeat number may assist in adhesion and in evasion of the host immune system (156). Some members lack a GPI anchor, such as *IFF11* in C. *albicans*, a secreted protein required for cell wall organization (5).

HYR1 is perhaps the best-studied family member in C. *albicans*. Expression is induced in hyphae, in azole-resistant cells, and when cells are engulfed by macrophages (4, 38, 79). *HYR1* is missing from the C. *dubliniensis* genome and appears to have been specifically deleted, leaving behind a small region from the 3′ end (73). The *HYR1* sequence in C. *albicans* WO-1 is a pseudogene containing one stop codon (http://cgob.ucd.ie); however, this may have arisen from errors during assembly. It is not clear if *HYR1* orthologs are present in the other *Candida* genomes. There are no syntenic orthologs (http://cgob.ucd.ie), but Jackson et al. (73) suggested that *HYR1* belongs to an old lineage.

PGA30 Family and an Uncharacterized Family

PGA30 was identified from C. *albicans* in a computational screen for GPI-anchored proteins (26). It is localized to the cell wall (15). There are 12 family members in C. *albicans*, 14 in C. *tropicalis*, and 6 each in C. *parapsilosis* and *L. elongisporus* (12). It is likely that this family plays important roles in cell wall organization in *Candida* species (also see chapter 14 for family gene functions).

A completely uncharacterized family (CF739) has nine members in C. *albicans* and C. *tropicalis*, seven in C. *dubliniensis*, and fewer than two in the other *Candida* species. This was designated the YFW family by Jones et al. (77) to indicate amino acid residues that are conserved with homologues in other fungal species. The biological role of this family is completely unknown.

The large-scale analysis of *Candida* genomes (12) confirmed that many of the multigene families described for C. *albicans* (9, 77) are also present in the genomes of other pathogenic *Candida* species. Secreted hydrolyases such as proteases, lipases, and phospholipases have been associated with pathogenesis in C. *albicans* (1, 67–69, 130, 142), as have lipases in C. *parapsilosis* (48). The OPT family may be necessary for adaptation to the host (122). In general, however, little experimental analysis has been performed in species other than C. *albicans*.

COMPARISON OF *C. ALBICANS* AND *C. DUBLINIENSIS*

The large-scale analysis by Butler et al. (12) identified gene family differences between *Candida* species. However, the analysis did not include the genome of C. *dubliniensis*, a very close relative of C. *albicans* that is substantially less pathogenic (52). Direct comparisons of these two species should increase our understanding of pathogenesis, particularly in C. *albicans*.

C. *albicans* and C. *dubliniensis* are the only two *Candida* species that generate true filaments, although C. *dubliniensis* is much less efficient (143, 157). Hyphal development is generally associated with virulence (61). Some of the species differences are caused by the inability of C. *dubliniensis* to regulate expression of the *NRG1* repressor (105). There are also substantial differences in the gene repertoires of the two species, however.

Moran et al. (104) used comparative genomic hybridization to compare C. *albicans* and C. *dubliniensis* and noted that several genes were missing or highly diverged in the latter species, including *HYR1* (discussed above), components of the biotin synthesis pathway, and members of the *HWP1* cell wall family. Direct comparison of the whole genome sequences revealed further differences (73).

In general, the C. *dubliniensis* genome has undergone substantial gene loss (73). There are 115 pseudogenes, of which 78 are intact in C. *albicans*. Of 16 filamentous growth regulator genes required for hyphal development in C. *albicans*, 8 are pseudogenes and 6 are absent from C. *dubliniensis*. The biggest differences in gene number are in the IFA family and the family of *TLO* transcription factors, both of which have undergone large expansions in C. *albicans*.

IFA proteins are rich in leucine repeats and have transmembrane domains. There are up to 33 IFA family members in C. *albicans*, only 1 in C. *tropicalis*, and no obvious homologues in most of the other *Candida* species. In C. *dubliniensis*, there are 21 loci corresponding to IFA genes; however, 14 of these are pseudogenes, and some have degenerated to such an extent that they are difficult to identify (73). There has therefore been a dramatic expansion of the IFA family in the C. *albicans* lineage, followed by subsequent loss in C. *dubliniensis*. The biological role of the family is not known. However, in C. *albicans*, family members differ at the C termini; some have acidic regions, and some are basic. It is therefore likely that the proteins function in different cellular pathways.

The first member of the *TLO* family (called *CTA2*) was fortuitously identified in a one-hybrid screen for transactivating factors and so is assumed to encode a transcription factor (78). van het Hoog et al. (155) subsequently showed that *CTA2* is part of a large gene family in C. *albicans*, containing at least 15 members. Most are located near the chromosome telomeres (one per chromosome arm, except for the right arm of chromosome 2 and the left arm of chromosome 7) and were renamed *TLO* genes (for telomere associated). One *TLO* gene (*TLO34*) is internal to chromosome 1.

There is only one *TLO* gene in most of the other *Candida* species, syntenic with *TLO2* at the right telomere of chromosome R in C. *albicans*. The C. *dubliniensis* genome has two *TLO* genes, one syntenic with *TLO2* and one (called *CdTLO1*) that is internal to chromosome 7 (73). Phylogenetic analysis indicates that the ancestral *TLO2* gene underwent a single duplication and transposition in C. *dubliniensis*, whereas in C. *albicans* the family has been significantly expanded. This differs mechanistically from the *IFA* family, which appears to have undergone a large expansion in the ancestor of C. *albicans* and C. *dubliniensis*, followed by gene loss in C. *dubliniensis*. Gene amplification may be associated with transposition, as most copies have an LTR kappa 5′ to the ORF (155). Six of the genes in C. *albicans* (*TLO5, -7, -8, -11, -13*, and *-16*) acquired an intron in the carboxyl domain (155). This presumably occurred during one duplication event and was conserved in later copies. There is evidence for differential splicing, suggesting that two different protein products are produced.

Deleting *CdTLO1* results in a major reduction in serum-induced hyphal formation in C. *dubliniensis* (73). The phenotype is complemented by introducing either *TLO11* or *TLO12* from C. *albicans*, indicating that the entire family may be involved in regulating hyphal morphogenesis.

The C. *albicans*-C. *dubliniensis* comparison has highlighted a potential role for the *IFA* and *TLO* families in pathogenesis. These have been understudied to date and are likely to be the focus of much future experimentation. The analysis also supports a view of C. *dubliniensis* as a defective pathogen, which has degenerated from a virulent ancestor mostly because of gene loss.

EVOLUTION OF THE MATING TYPE-LIKE LOCUS

The sequenced species of the CTG clade vary tremendously in their ability to mate (Table 1). *C. guilliermondii*, *C. lusitaniae*, *D. hansenii*, and *P. stipitis* belong to a subclade and are haploid and fully sexual. *P. stipitis* is homothallic (mating occurs between genetically identical isolates) and generates two hat-shaped spores through meiosis (101). *D. hansenii* (anamorph, *Candida famata*) is also homothallic and usually produces a small number of meiotic spores (154). In contrast, *C. guilliermondii* and *C. lusitaniae* (teleomorphs, *Pichia guilliermondii* and *Clavispora lusitaniae*) are heterothallic (distinct mating types).

Mating within the diploid *Candida* species is more complex. *C. albicans* was long assumed to be asexual, until the discovery of a mating type-like locus (*MTL*) (70). Mating takes place between diploid isolates that are homozygous at *MTL* (for **a** or α) (71, 92). Efficient mating requires a phenotypic switch from white to opaque cells, a phenomenon that is described in detail in chapter 6. Identical cells can also mate (homothallism) under certain conditions (2).

MTL idiomorphs contain regulatory genes (**a**1 and **a**2 in *MTL***a** and α1 and α2 in *MTL*α) and locus-specific alleles of poly(A) polymerase (*PAP*), phosphatidylinositol kinase (*PIK*), and oxysterol binding protein (*OBP*), with no known role in mating. Recruitment of *PAP*, *PIK*, and *OBP* to *MTL* is an ancient event that occurred in an ancestor of the CTG clade (12). Mating in *MTL***a** cells is regulated by the high–mobility-group domain protein **a**2, which has been lost from the *Saccharomyces* group (11, 150). The α-domain protein α1 regulates mating in *MTL*α cells, and in *MTL***a**/*MTL*α heterozygotes the homeodomain dimer **a**1/α2 regulates mating competency and white-opaque switching (149).

The organization of *MTL* in *C. dubliniensis* and *C. tropicalis* is very similar to that in *C. albicans* (Fig. 4), although there has been an inversion of the surrounding region in *C. dubliniensis* (10, 12, 73, 118). However, white-opaque switching is restricted to *C. albicans* and *C. dubliniensis* (149, 150, 164). Mating-competent forms of *C. dubliniensis* mate with opposite mating types and can also mate with *C. albicans* (118). Mating has not been observed in *C. tropicalis*.

To date, all isolates of *C. parapsilosis* tested contain only *MTL***a** idiomorphs, and *MTL***a***1* is a pseudogene (90). It therefore appears unlikely that this species can mate, even though the mating signal transduction pathway, from pheromones through MAP kinases to the transcription factor Cph1, is intact (12, 29). *L. elongisporus* was for a long time considered to be the sexual form of *C. parapsilosis* (74), but it is now clear that *L. elongisporus* is a distinct species related to *C. parapsilosis*, *C. orthopsilosis*, and *C. metapsilosis* (147).

L. elongisporus is generally assumed to be fully sexual and homothallic, although only one or two spores have been observed (120, 153). Astonishingly, analysis of the genome sequence shows that it does not encode any of the mating type regulators **a**1, **a**2, α1, and α2 (12). *PAP*, *OBP*, and *PIK* genes are adjacent to each other and in the same syntenic context as *MTL***a** in the other *Candida* species (Fig. 4). The *L. elongisporus* genome also does not encode the **a**-factor pheromone or the pheromone receptor (Table 4). The possibility that sexual reproduction occurs in this yeast is intriguing and deserving of more investigation.

The general structure of *MTL* is conserved in the haploid *Candida* clade, except that the homeodomain protein α2 is missing from all (Fig. 4). The heterothallic species *C. guilliermondii* is also missing **a**1 (121). Evolution of homothallism has occurred by two mechanisms in *P. stipitis* and *D. hansenii* (10, 12, 37). In the former, it is most likely that recombination between ancestral *MTL***a** and *MTL*α idiomorphs placed the regulatory genes (**a**1 and **a**2) together beside *OBP, PIK,* α1, and *PAP*. In *D. hansenii*, a similar recombination event placing the regulator genes together at *MTL***a** was probably followed by a second event separating **a**1, **a**2, and α1 from *PAP*, *OBP*, and *PIK* (37). In both species it appears that homothallism arose from heterothallic ancestors.

EVOLUTION OF MEIOSIS

Unused characters tend to decay over evolutionary time, although the rate of decay will depend upon the selection environment (60). Conservation of the mating loci, and the mating signal transduction pathway, is therefore generally taken as an indication that sexual reproduction does take place (62). This has been vindicated by the demonstration of sex in *Aspergillus fumigatus* (112). However, for other species, such as *C. glabrata*, a sexual cycle has not been described despite many attempts to find one (11, 107, 140, 161).

Conservation of a core set of meiotic genes, the "meiosis detection toolkit," is often used as a signal for sex (14, 131–133). True meiosis has not been observed in *C. albicans*; mating between diploid cells generates a tetraploid, which randomly loses chromosomes and in approximately 30% of cells reverts to diploidy (6). However, the core meiotic genes are conserved in the diploid *Candida* species, although it is possible that some (such as *SPO11*) have been recruited for other functions (43, 96, 151). A more detailed analysis revealed that the majority of genes associated with chromosome cohesion, meiosis, and recombination in fungi are conserved in *C. albicans* and any that are missing (such as *MLH2*) are also missing from the genomes of the sexual *Candida* species (Table 4) (12). The genomic evidence therefore suggests that meiosis may occur, albeit rarely.

Even in the sexual *Candida*, it is likely that meiosis and recombination are inefficient. Asci generally contain few spores (usually two), and some of the progeny are aneuploid (101, 121, 123, 154, 159). It is possible that meiosis has degenerated even further in the diploid species, resulting in the parasexual cycle that we observe in *C. albicans* today (43).

Interestingly, there has been some additional loss of meiosis-associated genes in the sexual species *C. guilliermondii* and *C. lusitaniae* (Table 4) (12). The genomes of these species do not encode components of the synaptonemal complex or the synapsis initiation complex and are also missing the Dmc1-dependent pathway for pairing of homologous proteins and the Msh4/Msh5 pathway for resolution of meiotic crossovers. These complexes are not always required for meiosis; Dmc1 pathway components are missing from *Drosophila* and *Caenorhabditis*, Msh4/5 is not present in *Drosophila*, and other fungi such as *Cryptococcus neoformans*, *Neurospora crassa*, and *Schizosaccharomyces pombe* do not form synaptonemal complexes (91, 115, 131). However, the extent of gene loss in *C. guilliermondii* and *C. lusitaniae* is unusual and suggests that we should reevaluate our model of meiosis in fungi. Most phylogenetic analyses infer that *C. guilliermondii* and *C. lusitaniae* are not each other's closest relatives (Fig. 1), suggesting that the losses of the genes occurred independently in the two species. It appears unlikely, however, that such massive genome arrangements would occur twice. However, some losses are shared with *D. hansenii* and may have occurred in a common ancestor (Table 4). We have noticed that the phylogenetic position of

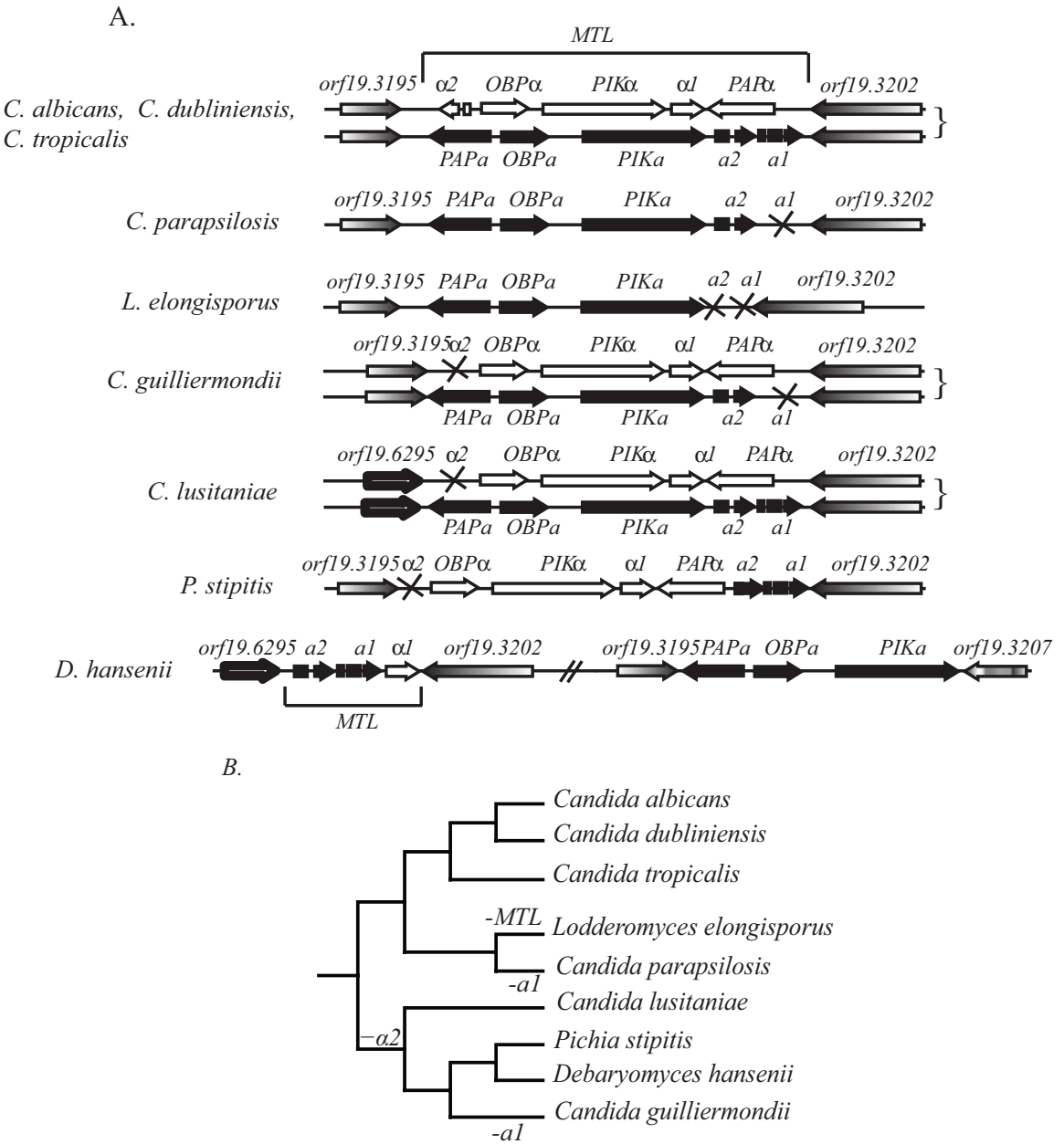

FIGURE 4 (A) Organization of MTL in *Candida* species. MTLα-specific genes are shown in white and MTL**a**-specific genes in black. C. *albicans* names are used for adjacent genes. Gene losses are indicated with crosses. Idiomorphs from heterothallic isolates are linked with braces. Genomic regions are not drawn to scale, and not all ORFs are shown. (B) Placement of gene losses on the phylogenetic tree. 10.1128/9781555817176.ch3f4

C. *lusitaniae* is difficult to assign with certainty and depends on the methods used. It is therefore possible that C. *guilliermondii* and C. *lusitaniae* are more closely related than current trees suggest.

HORIZONTAL GENE TRANSFER

Cross-species acquisition of genes has long been known to be important in the evolution of bacterial genomes (84, 145). The role of horizontal gene transfer (HGT) in the evolution of eukaryotes is still somewhat debatable and is the focus of much research. Major examples include the transfer of large numbers of genes from organelles such as mitochondria and plastids to the nuclear genomes of multicellular organisms, but this is a somewhat different event, termed endosymbiotic gene transfer (81, 85, 98). Identification of the acquisition of a small number of genes from bacteria or other sources is difficult and relies on the detection of phylogenetic incongruence, a deviation between the phylogenetic relationships inferred from a single gene with that proposed for the entire organism. Difficulties can arise when the phylogeny is in doubt. For example, an initial analysis of

TABLE 4 Loss of genes from mating signal transduction and meiotic pathways in *Candida* species[a]

Species	Mating signaling	Meiotic regulation	Synaptonemal complex formation	Meiotic recombination	Crossover resolution
L. elongisporus	**a**-Factor, *STE3*, *STE6*		*ZIP4*		
C. guilliermondii			*HOP1*, *MEK1*, *ZIP2*, *ZIP3*, *ZIP4*	*DMC1*, *HOP2*, *MND1*, *MEI5*	*MSH4*, *MSH5*, *MER3*
C. lusitaniae	*FAR1*		*HOP1*, *MEK1*, *ZIP2*, *ZIP3*, *ZIP4*	*DMC1*, *HOP2*, *MND1*, *MEI5*, *SAE3*	*MSH4*, *MSH5*, *MER3*
D. hansenii	**a**-Factor		*ZIP2*, *ZIP3*, *ZIP4*		*MSH4*, *MSH5*
Absent from all species[b]		*IME1*, *IDS2*, *SUM1*, *SPO12*	*ZIP1*, *RED1*	*MLH2*, *RAD55*, *REC104*, *REC114*, *SPO13*	

[a]Adapted from reference 12. The genes listed are *missing* from the relevant genomes.
[b]Including *C. albicans*, *C. dubliniensis*, *C. tropicalis*, and *C. parapsilosis*.

the human genome sequence suggested that around 100 genes were acquired from bacterial genomes (86), but most of these were resolved following further phylogenetic analysis (141). There are several well-characterized examples of gene transfer from bacteria to unicellular organisms such as protists. For example, rumen-dwelling ciliates are estimated to have acquired as much as 4% of their genome from bacteria, which may have allowed them to adapt to a very specific environment. Transfer of ATP transporters from *Chlamydia* to the genome of *Encephalitozoon cuniculi* has enabled the microsporidian to obtain ATP from the host (80). One of the best-characterized examples is the large-scale transfer of genes from bacteria, fungi, and plants to the genomes of bdelloid rotifers (53).

The abundance of whole genome sequences from fungi provides an ideal resource for the identification of HGT. In a recent analysis, Marcet-Houben and Gabaldon identified 713 examples of gene transfer from bacteria to the genomes of 60 fungi (97). These include the acquisition of a bacterial arsenate reductase in *Yarrowia lipolytica* and *Rhizopus oryzae* and transfer of catalases to plant-pathogenic fungi. Interestingly, by far the majority of the gene transfer events (65%) occur in the Pezizomycotina (the filamentous fungi), and much fewer were identified in the Saccharomycotina. The stringent approach of Marcet-Houben and Gabaldon likely underestimated the overall level of gene transfer; for example, they identified a single event in *S. cerevisiae*, whereas 12 genes were identified in earlier studies (124). However, the difference between the Pezizomycotina and the closely related Saccharomycotina is striking.

A search for HGT in *Candida* (CTG clade) species shows that it is an even rarer event in this group than in the rest of the Saccharomycotina (40). Prior to this analysis, only one example of a gene transfer into a CTG clade species had been identified—an apparent transfer of YdhR (a putative mono-oxygenase) from *Bacillus cereus* to *D. hansenii* (33). The analysis of Fitzpatrick et al. (40) identified two additional examples, both transfers of genes from bacterial sources into the genome of *C. parapsilosis*.

One of the genes identified encodes a proline racemase (PR) which was mostly likely acquired from a *Burkholderia* species, although the exact donor cannot be determined. The PR gene is present in the genome of *C. parapsilosis* and not in that of its close relative *L. elongisporus*, suggesting that it is a recent acquisition. Interestingly, the PR gene is found adjacent to a neutral amino acid transporter, in a region where synteny is not conserved with other *Candida* species (Fig. 5). Gene acquisition may therefore have been associated with a chromosomal recombination, possibly at a tRNA locus (Fig. 5). Fitzpatrick et al. (40) identified several examples of independent HGT of PR genes in the genomes of the Pezizomycotina, and Marcet-Houben and Gabaldon showed that other racemase genes are also transferred (97). The biological significance of these acquisitions is not clear, although they may be involved in the metabolism of D-isomers of amino acids, possibly for cell wall construction.

C. parapsilosis has also acquired a phenazine F (PhzF) homologue that most closely resembles a gene in *Photorhabdus luminescens* (39, 40). In bacteria, PhzF is part of an operon that is required for synthesis of phenazines, which are broad-spectrum antibiotics. PhzF is present in all the CTG clade species and several other Saccharomycotina, probably acquired from bacteria in an ancient horizontal transfer event. Interestingly, PhzF may also have been transferred from one of the CTG clade (most likely *D. hansenii*) to the genome of *Schizosaccharomyces pombe*, in a rare example of eukaryote-to-eukaryote HGT (40). However, the gene was lost in the last common ancestor of *C. parapsilosis* and *L. elongisporus* and reacquired more recently by *C. parapsilosis*. Again, the biological significance is unclear, but PhzF is likely to confer specific metabolic activities that are unknown at present.

Recent evidence suggests that fungal genomes may also acquire genes from nonretroviral RNA viruses (*Totovirus*) (148). Totoviruses infect many fungal species but are missing from those in the CTG clade (51). However, Taylor and Bruenn identified *Totovirus*-like genes in five fungal species, including *P. stipitis*, *D. hansenii*, and *C. parapsilosis*. Changes in the protein sequences of the fungal homologues suggest that they have been co-opted for different cellular functions. The change in the genetic code may have helped protect *Candida* species from infection by RNA-based viruses (148). It is has also been suggested that the CTG codon alteration blocks HGT in general (136), which may explain the small number of examples identified to date.

GENOME DATABASES

The growing body of genome information has made it difficult for individual scientists to keep track of what information and, indeed, what tools are available. One of the best sources of information is the *Candida* Genome Database (CGD) (http://www.candidagenome.org), which provides access to genome sequence data that are manually curated, as well as tools such as sequence comparison, data downloading, gene ontology analysis, and descriptions of biochemical pathways (137). The CGD is regularly updated

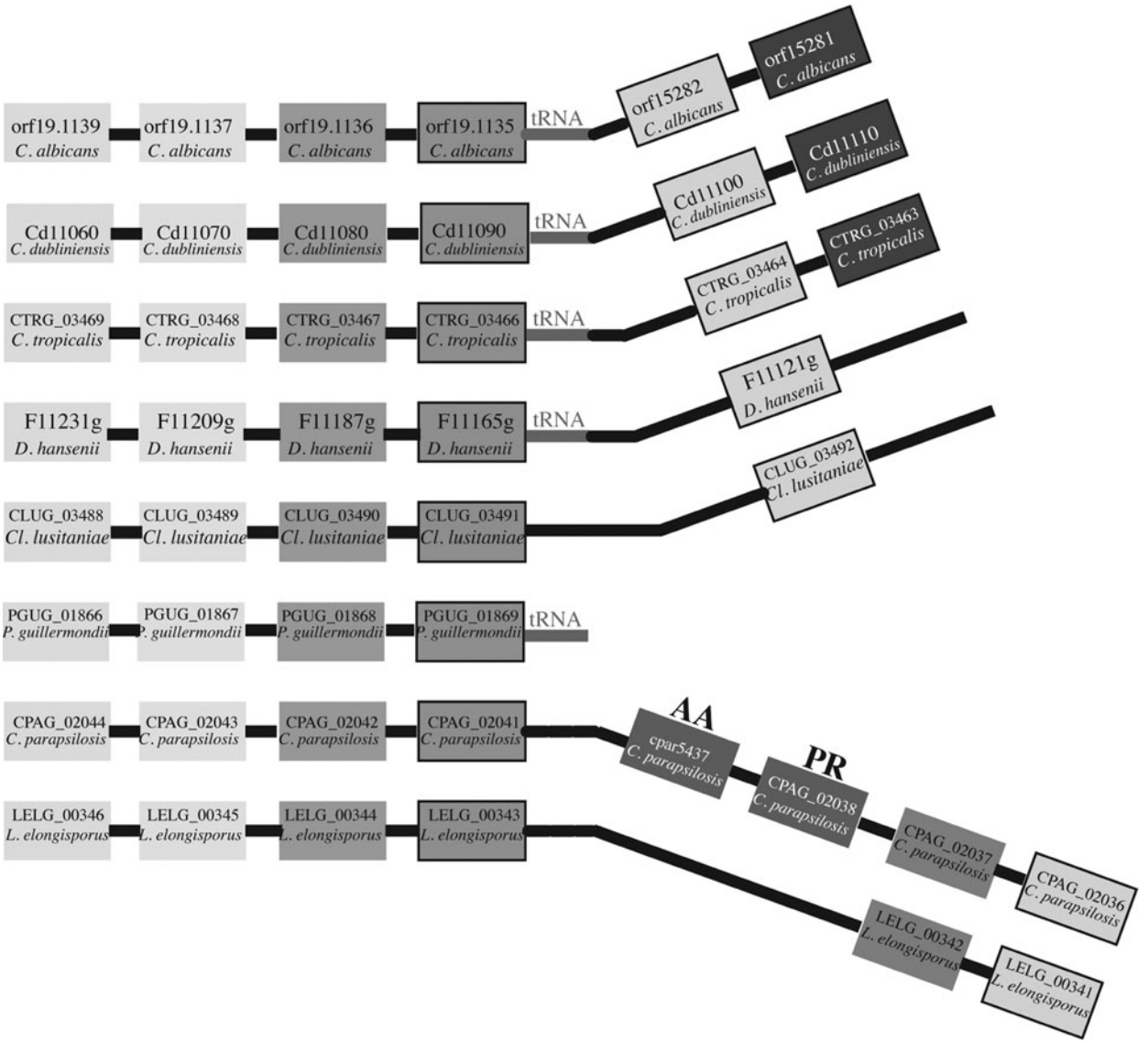

FIGURE 5 HGT of a proline racemase gene into C. *parapsilosis*. The species and gene names are shown in the individual boxes. *Clavispora lusitaniae* is equivalent to C. *lusitaniae*, and *P. guilliermondii* is equivalent to C. *guilliermondii*. Orthologous genes are stacked in columns, or pillars. Gene order is conserved on the left-hand side; on the right-hand side synteny between C. *parapsilosis* and *L. elongisporus* is conserved, except for an insertion of a PR and a neutral amino acid transporter (AA) in C. *parapsilosis*. There is no conservation in synteny in this region between C. *parapsilosis*/*L. elongisporus* and the other *Candida* species. Reproduced from reference 40 with permission. 10.1128/9781555817176.ch3f5

and corrected. The database concentrates mainly on C. *albicans*, although links to C. *dubliniensis* orthologs are provided. Comparative genomic tools for *Candida* species are available from the Broad Institute (Fungal Genomics Initiative), the Pasteur Institute (CandidaDB), and University College Dublin (*Candida* Gene Order Browser).

The Fungal Genome Initiative provides access to approximately 50 fungal genomes, including those of the *Candida* species described by Butler et al. (12) (http://www.broadinstitute.org/annotation/genome/candida_group/MultiHome.html). The site provides downloads of protein predictions, sequences upstream and downstream from predicted ORFs, and data sets of SNPs. Information on gene families is also available. Tools provided include genome browsers, BLAST searches, and some comparative analyses.

CandidaDB (http://genodb.pasteur.fr/cgi-bin/WebObjects/CandidaDB) began as a database for C. *albicans* SC5314 (27) and has been now been expanded to include C. *albicans* WO-1, C. *tropicalis*, C. *lusitaniae*, C. *guilliermondii*, *L. elongisporus*, *D. hansenii*, *P. stipitis*, and a reference genome from *S. cerevisiae* (126). The database includes genome browsers and provides information on structural features and functional domains. All data are searchable, making it easy to identify and download orthologs from all species. Precomputed BLASTP and protein family tools are included.

CGOB (http://cgob.ucd.ie) is an online tool designed to help visualize and analyze synteny (gene order) in *Candida* genomes (42). The browser includes genomes of all the sequenced CTG clade species (10 genomes) plus the reference species *S. cerevisiae*. The genome annotations were manually edited, resulting in merging of a total of 694 partial genes to yield 327 full-length proteins. The browser was used to identify clusters of metabolic genes, such as those required for biotin synthesis. The *C. albicans* genome contains a cluster of four biotin genes, of which three are missing from *C. dubliniensis*. The remaining species contain some genes required for biotin synthesis, but the pathway is not intact in any.

Because *C. glabrata* is distantly related to the other *Candida* species, it is not included in the databases described above. Comparative analysis of 18 genomes from the Saccharomycotina (including *C. glabrata*) is supported by the Génolevures database (http://www.genolevures.org/) (135). Analysis of gene order and of genome structure is facilitated by the Yeast Gene Order Browser (http://wolfe.gen.tcd.ie/ygob/) (13).

The website http://fungalgenomes.org, maintained by Jason Stajich at UC Riverside, is an excellent resource for monitoring fungal genome projects.

In conclusion, analysis of the vast array of genomic data available has already made, and is likely to continue to make, major contributions to our understanding of the biology of *Candida* species. Future directions are likely to include the sequencing of additional genomes (such as that of *C. krusei*) and the application of next-generation sequencing to numerous isolates of the same species. We are truly in the midst of an exciting era of comparative genome analysis.

REFERENCES

1. **Albrecht, A., A. Felk, I. Pichova, J. R. Naglik, M. Schaller, P. de Groot, D. MacCallum, F. C. Odds, W. Schafer, F. Klis, M. Monod, and B. Hube.** 2006. Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. *J. Biol. Chem.* **281:**688–694.
2. **Alby, K., D. Schaefer, and R. J. Bennett.** 2009. Homothallic and heterothallic mating in the opportunistic pathogen *Candida albicans*. *Nature* **460:**890–893.
3. **Almeida, R. S., S. Brunke, A. Albrecht, S. Thewes, M. Laue, J. E. Edwards, S. G. Filler, and B. Hube.** 2008. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog.* **4:**e1000217.
4. **Bailey, D. A., P. J. Feldmann, M. Bovey, N. A. Gow, and A. J. Brown.** 1996. The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J. Bacteriol.* **178:**5353–5360.
5. **Bates, S., J. M. de la Rosa, D. M. MacCallum, A. J. Brown, N. A. Gow, and F. C. Odds.** 2007. *Candida albicans* Iff11, a secreted protein required for cell wall structure and virulence. *Infect. Immun.* **75:**2922–2928.
6. **Bennett, R. J., and A. D. Johnson.** 2003. Completion of a parasexual cycle in *Candida albicans* by induced chromosome loss in tetraploid strains. *EMBO J.* **22:**2505–2515.
7. **Bennett, R. J., and A. D. Johnson.** 2004. Mating in *Candida albicans* and the search for a sexual cycle. *Annu. Rev. Microbiol.* **59:**233–255.
8. **Box, J. A., J. T. Bunch, W. Tang, and P. Baumann.** 2008. Spliceosomal cleavage generates the 3′ end of telomerase RNA. *Nature* **456:**910–914.
9. **Braun, B. R., M. van Het Hoog, C. d'Enfert, M. Martchenko, J. Dungan, A. Kuo, D. O. Inglis, M. A. Uhl, H. Hogues, M. Berriman, M. Lorenz, A. Levitin, U. Oberholzer, C. Bachewich, D. Harcus, A. Marcil, D. Dignard, T. Iouk, R. Zito, L. Frangeul, F. Tekaia, K. Rutherford, E. Wang, C. A. Munro, S. Bates, N. A. Gow, L. L. Hoyer, G. Kohler, J. Morschhauser, G. Newport, S. Znaidi, M. Raymond, B. Turcotte, G. Sherlock, M. Costanzo, J. Ihmels, J. Berman, D. Sanglard, N. Agabian, A. P. Mitchell, A. D. Johnson, M. Whiteway, and A. Nantel.** 2005. A human-curated annotation of the *Candida albicans* genome. *PLoS Genet.* 1:e1.
10. **Butler, G.** 2010. Fungal sex and pathogenesis. *Clin. Microbiol. Rev.* **23:**140–159.
11. **Butler, G., C. Kenny, A. Fagan, C. Kurischko, C. Gaillardin, and K. H. Wolfe.** 2004. Evolution of the *MAT* locus and its Ho endonuclease in yeast species. *Proc. Natl Acad. Sci. USA* **101:**1632–1637.
12. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J. L. Reedy, I. Agrafioti, M. B. Arnaud, S. Bates, A. J. Brown, S. Brunke, M. C. Costanzo, D. A. Fitzpatrick, P. W. de Groot, D. Harris, L. L. Hoyer, B. Hube, F. M. Klis, C. Kodira, N. Lennard, M. E. Logue, R. Martin, A. M. Neiman, E. Nikolaou, M. A. Quail, J. Quinn, M. C. Santos, F. F. Schmitzberger, G. Sherlock, P. Shah, K. A. Silverstein, M. S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M. P. Stumpf, P. E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N. A. Gow, M. C. Lorenz, B. W. Birren, M. Kellis, and C. A. Cuomo.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459:**657–662.
13. **Byrne, K. P., and K. H. Wolfe.** 2005. The Yeast Gene Order Browser: combining curated homology and syntenic context reveals gene fate in polyploid species. *Genome Res.* **15:**1456–1461.
14. **Carr, M., B. S. Leadbeater, and S. L. Baldauf.** 2010. Conserved meiotic genes point to sex in the choanoflagellates. *J. Eukaryot. Microbiol.* **57:**56–62.
15. **Castillo, L., E. Calvo, A. I. Martinez, J. Ruiz-Herrera, E. Valentin, J. A. Lopez, and R. Sentandreu.** 2008. A study of the *Candida albicans* cell wall proteome. *Proteomics* **8:**3871–3881.
16. **Cha, R. S., and N. Kleckner.** 2002. ATR homolog Mec1 promotes fork progression, thus averting breaks in replication slow zones. *Science* **297:**602–606.
17. **Chibana, H., J. L. Beckerman, and P. T. Magee.** 2000. Fine-resolution physical mapping of genomic diversity in *Candida albicans*. *Genome Res.* **10:**1865–1877.
18. **Chibana, H., S. Iwaguchi, M. Homma, A. Chindamporn, Y. Nakagawa, and K. Tanaka.** 1994. Diversity of tandemly repetitive sequences due to short periodic repetitions in the chromosomes of *Candida albicans*. *J. Bacteriol.* **176:**3851–3858.
19. **Chindamporn, A., Y. Nakagawa, I. Mizuguchi, H. Chibana, M. Doi, and K. Tanaka.** 1998. Repetitive sequences (RPSs) in the chromosomes of *Candida albicans* are sandwiched between two novel stretches, HOK and RB2, common to each chromosome. *Microbiology* **144:**849–857.
20. **Chu, W. S., B. B. Magee, and P. T. Magee.** 1993. Construction of an SfiI macrorestriction map of the *Candida albicans* genome. *J. Bacteriol.* **175:**6637–6651.
21. **Codon, A. C., T. Benitez, and M. Korhola.** 1998. Chromosomal polymorphism and adaptation to specific industrial environments of *Saccharomyces* strains. *Appl. Microbiol. Biotechnol.* **49:**154–163.
22. **Coste, A., A. Selmecki, A. Forche, D. Diogo, M. E. Bougnoux, C. d'Enfert, J. Berman, and D. Sanglard.**

2007. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. *Eukaryot. Cell* **6:**1889–1904.
23. **Coste, A. T., V. Turner, F. Ischer, J. Morschhauser, A. Forche, A. Selmecki, J. Berman, J. Bille, and D. Sanglard.** 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics* **172:**2139–2156.
24. **Daniels, K. J., S. R. Lockhart, J. F. Staab, P. Sundstrom, and D. R. Soll.** 2003. The adhesin Hwp1 and the first daughter cell localize to the **a/a** portion of the conjugation bridge during *Candida albicans* mating. *Mol. Biol. Cell* **14:**4920–4930.
25. **de Groot, P. W., A. D. de Boer, J. Cunningham, H. L. Dekker, L. de Jong, K. J. Hellingwerf, C. de Koster, and F. M. Klis.** 2004. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryot. Cell* **3:**955–965.
26. **De Groot, P. W., K. J. Hellingwerf, and F. M. Klis.** 2003. Genome-wide identification of fungal GPI proteins. *Yeast* **20:**781–796.
27. **d'Enfert, C., S. Goyard, S. Rodriguez-Arnaveilhe, L. Frangeul, L. Jones, F. Tekaia, O. Bader, A. Albrecht, L. Castillo, A. Dominguez, J. F. Ernst, C. Fradin, C. Gaillardin, S. Garcia-Sanchez, P. de Groot, B. Hube, F. M. Klis, S. Krishnamurthy, D. Kunze, M. C. Lopez, A. Mavor, N. Martin, I. Moszer, D. Onesime, J. Perez Martin, R. Sentandreu, E. Valentin, and A. J. Brown.** 2005. CandidaDB: a genome database for *Candida albicans* pathogenomics. *Nucleic Acids Res.* **33:**D353–D357.
28. **Deshpande, A. M., and C. S. Newlon.** 1996. DNA replication fork pause sites dependent on transcription. *Science* **272:**1030–1033.
29. **Dignard, D., A. L. El-Naggar, M. E. Logue, G. Butler, and M. Whiteway.** 2007. Identification and characterization of *MFA1*, the gene encoding *Candida albicans* **a**-factor pheromone. *Eukaryot. Cell* **6:**487–494.
30. **Diogo, D., C. Bouchier, C. d'Enfert, and M. E. Bougnoux.** 2009. Loss of heterozygosity in commensal isolates of the asexual diploid yeast *Candida albicans*. *Fungal Genet. Biol.* **46:**159–168.
31. **Di Rienzi, S. C., D. Collingwood, M. K. Raghurama, and B. J. Brewer.** 2009. Fragile genomic sites are associated with origins of replication. *Gen. Biol. Evol.* **2009:**350–363.
32. **Dranginis, A. M., J. M. Rauceo, J. E. Coronado, and P. N. Lipke.** 2007. A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. *Microbiol. Mol. Biol. Rev.* **71:**282–294.
33. **Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. De Montigny, C. Marck, C. Neuveglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J. M. Beckerich, E. Beyne, C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confanioleri, A. De Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J. M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G. F. Richard, M. L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J. L. Soucciet.** 2004. Genome evolution in yeasts. *Nature* 430:35–44.
34. **Dunkel, N., J. Blass, P. D. Rogers, and J. Morschhauser.** 2008. Mutations in the multi-drug resistance regulator *MRR1*, followed by loss of heterozygosity, are the main cause of *MDR1* overexpression in fluconazole-resistant *Candida albicans* strains. *Mol. Microbiol.* **69:**827–840.
35. **Dunn, B., R. P. Levine, and G. Sherlock.** 2005. Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. *BMC Genomics* **6:**53.
36. **Ene, I. V., and R. J. Bennett.** 2009. Hwp1 and related adhesins contribute to both mating and biofilm formation in *Candida albicans*. *Eukaryot. Cell* **8:**1909–1913.
37. **Fabre, E., H. Muller, P. Therizols, I. Lafontaine, B. Dujon, and C. Fairhead.** 2005. Comparative genomics in hemiascomycete yeasts: evolution of sex, silencing, and subtelomeres. *Mol. Biol. Evol.* **22:**856–873.
38. **Fernandez-Arenas, E., V. Cabezon, C. Bermejo, J. Arroyo, C. Nombela, R. Diez-Orejas, and C. Gil.** 2007. Integrated proteomics and genomics strategies bring new insight into *Candida albicans* response upon macrophage interaction. *Mol. Cell. Proteomics* **6:**460–478.
39. **Fitzpatrick, D. A., and G. Butler.** 2010. Comparative genomic analysis of pathogenic yeasts and the evolution of virulence, p. 1–18. *In* H. R. Ashbee and E. Bignell (ed.), *Pathogenic Yeasts*. Springer, Heidelberg, Germany.
40. **Fitzpatrick, D. A., M. E. Logue, and G. Butler.** 2008. Evidence of recent interkingdom horizontal gene transfer between bacteria and *Candida parapsilosis*. *BMC Evol. Biol.* **8:**181.
41. **Fitzpatrick, D. A., M. E. Logue, J. E. Stajich, and G. Butler.** 2006. A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. *BMC Evol. Biol.* **6:**99.
42. **Fitzpatrick, D. A., P. O'Gaora, K. P. Byrne, and G. Butler.** 2010. Analysis of gene evolution and metabolic pathways using the Candida Gene Order Browser. *BMC Genomics* **11:**290.
43. **Forche, A., K. Alby, D. Schaefer, A. D. Johnson, J. Berman, and R. J. Bennett.** 2008. The parasexual cycle in *Candida albicans* provides an alternative pathway to meiosis for the formation of recombinant strains. *PLoS Biol.* **6:**e110.
44. **Forche, A., P. T. Magee, A. Selmecki, J. Berman, and G. May.** 2009. Evolution in *Candida albicans* populations during a single passage through a mouse host. *Genetics* **182:**799–811.
45. **Forche, A., G. May, and P. T. Magee.** 2005. Demonstration of loss of heterozygosity by single-nucleotide polymorphism microarray analysis and alterations in strain morphology in *Candida albicans* strains during infection. *Eukaryot. Cell* **4:**156–165.
46. **Fu, Y., A. S. Ibrahim, D. C. Sheppard, Y. C. Chen, S. W. French, J. E. Cutler, S. G. Filler, and J. E. Edwards, Jr.** 2002. *Candida albicans* Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. *Mol. Microbiol.* **44:**61–72.
47. **Fu, Y., G. Rieg, W. A. Fonzi, P. H. Belanger, J. E. Edwards, Jr., and S. G. Filler.** 1998. Expression of the *Candida albicans* gene *ALS1* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells. *Infect. Immun.* **66:**1783–1786.
48. **Gácser, A., D. Trofa, W. Schäfer, and J. D. Nosanchuk.** 2007. Targeted gene deletion in *Candida parapsilosis* demonstrates the role of secreted lipase in virulence. *J. Clin. Investig.* **117:**3049–3058.
49. **Garcia-Sanchez, S., S. Aubert, I. Iraqui, G. Janbon, J. M. Ghigo, and C. D'Enfert.** 2004. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot. Cell* **3:**536–545.
50. **Gaur, N. K., and S. A. Klotz.** 1997. Expression, cloning, and characterization of a *Candida albicans* gene, *ALA1*, that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins. *Infect. Immun.* **65:**5289–5294.

51. **Ghabrial, S.** 2008. Totiviruses, p. 163–174. *In* B. W. J. Mahy and M. H. V. van Regenmortel (ed.), *Encyclopedia of Virology*, 3rd ed., vol. 5. Academic Press, New York, NY.
52. **Gilfillan, G. D., D. J. Sullivan, K. Haynes, T. Parkinson, D. C. Coleman, and N. A. Gow.** 1998. *Candida dubliniensis*: phylogeny and putative virulence factors. *Microbiology* **144:**829–838.
53. **Gladyshev, E. A., M. Meselson, and I. R. Arkhipova.** 2008. Massive horizontal gene transfer in bdelloid rotifers. *Science* **320:**1210–1213.
54. **Goffeau, A., B. G. Barrell, H. Bussey, R. W. Davis, B. Dujon, H. Feldmann, F. Galibert, J. D. Hoheisel, C. Jacq, M. Johnston, E. J. Louis, H. W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, and S. G. Oliver.** 1996. Life with 6000 genes. *Science* **274:**546, 563–567.
55. **Goodwin, T. J., D. E. Dalle Nogare, M. I. Butler, and R. T. Poulter.** 2003. Ty3/gypsy-like retrotransposons in *Candida albicans* and *Candida dubliniensis*: Tca3 and Tcd3. *Yeast* **20:**493–508.
56. **Goodwin, T. J., J. E. Ormandy, and R. T. Poulter.** 2001. L1-like non-LTR retrotransposons in the yeast *Candida albicans*. *Curr. Genet.* **39:**83–91.
57. **Goodwin, T. J., and R. T. Poulter.** 2000. Multiple LTR-retrotransposon families in the asexual yeast *Candida albicans*. *Genome Res.* **10:**174–191.
58. **Goodwin, T. J., and R. T. Poulter.** 1998. The CARE-2 and rel-2 repetitive elements of *Candida albicans* contain LTR fragments of a new retrotransposon. *Gene* **218:**85–93.
59. **Gunisova, S., E. Elboher, J. Nosek, V. Gorkovoy, Y. Brown, J. F. Lucier, N. Laterreur, R. J. Wellinger, Y. Tzfati, and L. Tomaska.** 2009. Identification and comparative analysis of telomerase RNAs from *Candida* species reveal conservation of functional elements. *RNA* 15:546–559.
60. **Hall, A. R., and N. Colegrave.** 2008. Decay of unused characters by selection and drift. *J. Evol. Biol.* **21:**610–617.
61. **Hall, R. A., F. Cottier, and F. A. Muhlschlegel.** 2009. Molecular networks in the fungal pathogen *Candida albicans*. *Adv. Appl. Microbiol.* **67:**191–212.
62. **Heitman, J.** 2006. Sexual reproduction and the evolution of microbial pathogens. *Curr. Biol.* **16:**R711–R725.
63. **Hieter, P., D. Pridmore, J. H. Hegemann, M. Thomas, R. W. Davis, and P. Philippsen.** 1985. Functional selection and analysis of yeast centromeric DNA. *Cell* 42:913–921.
64. **Hoyer, L. L.** 2001. The ALS gene family of *Candida albicans*. *Trends Microbiol.* **9:**176–180.
65. **Hoyer, L. L., C. B. Green, S. H. Oh, and X. Zhao.** 2008. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (*ALS*) gene family—a sticky pursuit. *Med. Mycol.* **46:**1–15.
66. **Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi.** 1995. *Candida albicans ALS1*: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. *Mol. Microbiol.* **15:**39–54.
67. **Hruskova-Heidingsfeldova, O.** 2008. Secreted proteins of *Candida albicans*. *Frontiers Biosci.* **13:**7227–7242.
68. **Hube, B., D. Sanglard, F. C. Odds, D. Hess, M. Monod, W. Schafer, A. J. Brown, and N. A. Gow.** 1997. Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. *Infect. Immun.* **65:**3529–3538.
69. **Hube, B., F. Stehr, M. Bossenz, A. Mazur, M. Kretschmar, and W. Schafer.** 2000. Secreted lipases of *Candida albicans*: cloning, characterisation and expression analysis of a new gene family with at least ten members. *Arch. Microbiol.* **174:**362–374.
70. **Hull, C. M., and A. D. Johnson.** 1999. Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. *Science* **285:**1271–1275.
71. **Hull, C. M., R. M. Raisner, and A. D. Johnson.** 2000. Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. *Science* **289:**307–310.
72. **Iwaguchi, S., M. Homma, H. Chibana, and K. Tanaka.** 1992. Isolation and characterization of a repeated sequence (RPS1) of *Candida albicans*. *J. Gen. Microbiol.* **138:**1893–1900.
73. **Jackson, A. P., J. A. Gamble, T. Yeomans, G. P. Moran, D. Saunders, D. Harris, M. Aslett, J. F. Barrell, G. Butler, F. Citiulo, D. C. Coleman, P. W. de Groot, T. J. Goodwin, M. A. Quail, J. McQuillan, C. A. Munro, A. Pain, R. T. Poulter, M. A. Rajandream, H. Renauld, M. J. Spiering, A. Tivey, N. A. Gow, B. Barrell, D. J. Sullivan, and M. Berriman.** 2009. Comparative genomics of the fungal pathogens *Candida dubliniensis* and *C. albicans*. *Genome Res.* **19:**2231–2244.
74. **James, S. A., M. D. Collins, and I. N. Roberts.** 1994. The genetic relationship of *Lodderomyces elongisporus* to other ascomycete yeast species as revealed by small-subunit rRNA gene sequences. *Lett. Appl. Microbiol.* **19:**308–311.
75. **Jeffries, T. W., I. V. Grigoriev, J. Grimwood, J. M. Laplaza, A. Aerts, A. Salamov, J. Schmutz, E. Lindquist, P. Dehal, H. Shapiro, Y. S. Jin, V. Passoth, and P. M. Richardson.** 2007. Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat. Biotechnol.* **25:**319–326.
76. **Joly, S., C. Pujol, and D. R. Soll.** 2002. Microevolutionary changes and chromosomal translocations are more frequent at RPS loci in *Candida dubliniensis* than in *Candida albicans*. *Infect. Genet. Evol.* **2:**19–37.
77. **Jones, T., N. A. Federspiel, H. Chibana, J. Dungan, S. Kalman, B. B. Magee, G. Newport, Y. R. Thorstenson, N. Agabian, P. T. Magee, R. W. Davis, and S. Scherer.** 2004. The diploid genome sequence of *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **101:**7329–7334.
78. **Kaiser, B., T. Munder, H. P. Saluz, W. Kunkel, and R. Eck.** 1999. Identification of a gene encoding the pyruvate decarboxylase gene regulator CaPdc2p from *Candida albicans*. *Yeast* **15:**585–591.
79. **Karababa, M., A. T. Coste, B. Rognon, J. Bille, and D. Sanglard.** 2004. Comparison of gene expression profiles of *Candida albicans* azole-resistant clinical isolates and laboratory strains exposed to drugs inducing multidrug transporters. *Antimicrob. Agents Chemother.* **48:**3064–3079.
80. **Katinka, M. D., S. Duprat, E. Cornillot, G. Metenier, F. Thomarat, G. Prensier, V. Barbe, E. Peyretaillade, P. Brottier, P. Wincker, F. Delbac, H. El Alaoui, P. Peyret, W. Saurin, M. Gouy, J. Weissenbach, and C. P. Vivares.** 2001. Genome sequence and gene compaction of the eukaryote parasite *Encephalitozoon cuniculi*. *Nature* **414:**450–453.
81. **Keeling, P. J., and J. D. Palmer.** 2008. Horizontal gene transfer in eukaryotic evolution. *Nat. Rev. Genet.* **9:**605–618.
82. **Ketel, C., H. S. Wang, M. McClellan, K. Bouchonville, A. Selmecki, T. Lahav, M. Gerami-Nejad, and J. Berman.** 2009. Neocentromeres form efficiently at multiple possible loci in *Candida albicans*. *PLoS Genet.* **5:**e1000400.
83. **Kitada, K., E. Yamaguchi, K. Hamada, and M. Arisawa.** 1997. Structural analysis of a *Candida glabrata* centromere and its functional homology to the *Saccharomyces cerevisiae* centromere. *Curr. Genet.* **31:**122–127.
84. **Koonin, E. V., and Y. I. Wolf.** 2008. Genomics of bacteria and archaea: the emerging dynamic view of the prokaryotic world. *Nucleic Acids Res.* **36:**6688–6719.
85. **Kurland, C. G., and S. G. Andersson.** 2000. Origin and evolution of the mitochondrial proteome. *Microbiol. Mol. Biol. Rev.* **64:**786–820.

86. **Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. LeVine, P. McEwan, K. McKernan, J. Meldrim, J. P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J. C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R. H. Waterston, R. K. Wilson, L. W. Hillier, J. D. McPherson, M. A. Marra, E. R. Mardis, L. A. Fulton, A. T. Chinwalla, K. H. Pepin, W. R. Gish, S. L. Chissoe, M. C. Wendl, K. D. Delehaunty, T. L. Miner, A. Delehaunty, J. B. Kramer, L. L. Cook, R. S. Fulton, D. L. Johnson, P. J. Minx, S. W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J. F. Cheng, A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, et al.** 2001. Initial sequencing and analysis of the human genome. *Nature* **409:**860–921.
87. **Lepingle, A., S. Casaregola, C. Neuveglise, E. Bon, H. Nguyen, F. Artiguenave, P. Wincker, and C. Gaillardin.** 2000. Genomic exploration of the hemiascomycetous yeasts: 14. *Debaryomyces hansenii* var. *hansenii*. *FEBS Lett*. **487:**82–86.
88. **Lockhart, S. R., S. A. Messer, M. A. Pfaller, and D. J. Diekema.** 2008. *Lodderomyces elongisporus* masquerading as *Candida parapsilosis* as a cause of bloodstream infections. *J. Clin. Microbiol*. **46:**374–376.
89. **Lockhart, S. R., C. Pujol, K. J. Daniels, M. G. Miller, A. D. Johnson, M. A. Pfaller, and D. R. Soll.** 2002. In *Candida albicans*, white-opaque switchers are homozygous for mating type. *Genetics* **162:**737–745.
90. **Logue, M. E., S. Wong, K. H. Wolfe, and G. Butler.** 2005. A genome sequence survey shows that the pathogenic yeast *Candida parapsilosis* has a defective *MTL***a***1* allele at its mating type locus. *Eukaryot. Cell* **4:**1009–1017.
91. **Lorenz, A., J. L. Wells, D. W. Pryce, M. Novatchkova, F. Eisenhaber, R. J. McFarlane, and J. Loidl.** 2004. *S. pombe* meiotic linear elements contain proteins related to synaptonemal complex components. *J. Cell Sci*. **117:**3343–3351.
92. **Magee, B. B., and P. T. Magee.** 2000. Induction of mating in *Candida albicans* by construction of *MTL***a** and *MTL*α strains. *Science* **289:**310–313.
93. **Magee, B. B., M. D. Sanchez, D. Saunders, D. Harris, M. Berriman, and P. T. Magee.** 2008. Extensive chromosome rearrangements distinguish the karyotype of the hypovirulent species *Candida dubliniensis* from the virulent *Candida albicans*. *Fungal Genet. Biol*. **45:**338–350.
94. **Magee, P. T., and H. Chibana.** 2002. The genomes of *Candida albicans* and other *Candida* species, p. 293–304. *In* R. Calderone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
95. **Malik, H. S., and S. Henikoff.** 2009. Major evolutionary transitions in centromere complexity. *Cell* **138:** 1067–1082.
96. **Malik, S., A. Pightling, L. Stefaniak, A. Schurko, and J. Logsdon.** 2008. An expanded inventory of conserved meiotic genes provides evidence for sex in *Trichomonas vaginalis*. *PLoS One* **3:**e2879.
97. **Marcet-Houben, M., and T. Gabaldon.** 2009. Acquisition of prokaryotic genes by fungal genomes. *Trends Genet*. **26:**5–8.
98. **Martin, W., T. Rujan, E. Richly, A. Hansen, S. Cornelsen, T. Lins, D. Leister, B. Stoebe, M. Hasegawa, and D. Penny.** 2002. Evolutionary analysis of *Arabidopsis*, cyanobacterial, and chloroplast genomes reveals plastid phylogeny and thousands of cyanobacterial genes in the nucleus. *Proc. Natl. Acad. Sci. USA* **99:**12246–12251.
99. **McEachern, M. J., and E. H. Blackburn.** 1994. A conserved sequence motif within the exceptionally diverse telomeric sequences of budding yeasts. *Proc. Natl. Acad. Sci. USA* **91:**3453–3457.
100. **McEachern, M. J., and J. B. Hicks.** 1993. Unusually large telomeric repeats in the yeast *Candida albicans*. *Mol. Cell. Biol*. **13:**551–560.
101. **Melake, T., V. V. Passoth, and U. Klinner.** 1996. Characterization of the genetic system of the xylose-fermenting yeast *Pichia stipitis*. *Curr. Microbiol*. **33:**237–242.
102. **Miller, M. G., and A. D. Johnson.** 2002. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell* **110:**293–302.
103. **Mishra, P. K., M. Baum, and J. Carbon.** 2007. Centromere size and position in *Candida albicans* are evolutionarily conserved independent of DNA sequence heterogeneity. *Mol. Genet. Genomics* **278:**455–465.
104. **Moran, G., C. Stokes, S. Thewes, B. Hube, D. C. Coleman, and D. Sullivan.** 2004. Comparative genomics using *Candida albicans* DNA microarrays reveals absence and divergence of virulence-associated genes in *Candida dubliniensis*. *Microbiology* **150:**3363–3382.
105. **Moran, G. P., D. M. MacCallum, M. J. Spiering, D. C. Coleman, and D. J. Sullivan.** 2007. Differential regulation of the transcriptional repressor *NRG1* accounts for altered host-cell interactions in *Candida albicans* and *Candida dubliniensis*. *Mol. Microbiol*. **66:**915–929.
106. **Moreno-Ruiz, E., M. Galan-Diez, W. Zhu, E. Fernandez-Ruiz, C. d'Enfert, S. G. Filler, P. Cossart, and E. Veiga.** 2009. *Candida albicans* internalization by host cells is mediated by a clathrin-dependent mechanism. *Cell. Microbiol*. **11:**1179–1189.
107. **Muller, H., C. Hennequin, J. Gallaud, B. Dujon, and C. Fairhead.** 2008. The asexual yeast *Candida glabrata* maintains distinct **a** and α haploid mating types. *Eukaryot. Cell* **7:**848–858.
108. **Naglik, J. R., S. J. Challacombe, and B. Hube.** 2003. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev*. **67:**400–428.
109. **Nailis, H., R. Vandenbroucke, K. Tilleman, D. Deforce, H. Nelis, and T. Coenye.** 2009. Monitoring *ALS1* and *ALS3* gene expression during *in vitro Candida albicans* biofilm formation under continuous flow conditions. *Mycopathologia* **167:**9–17.
110. **Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell.** 2006. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation *in vitro* and *in vivo*. *PLoS Pathog*. **2:**e63.
111. **Nobile, C. J., H. A. Schneider, J. E. Nett, D. C. Sheppard, S. G. Filler, D. R. Andes, and A. P. Mitchell.** 2008. Complementary adhesin function in *C. albicans* biofilm formation. *Curr. Biol*. **18:**1017–1024.
112. **O'Gorman, C. M., H. T. Fuller, and P. S. Dyer.** 2009. Discovery of a sexual cycle in the opportunistic fungal pathogen *Aspergillus fumigatus*. *Nature* **457:**471–474.
113. **Ohama, T., T. Suzuki, M. Mori, S. Osawa, T. Ueda, K. Watanabe, and T. Nakase.** 1993. Non-universal decoding of the leucine codon CUG in several *Candida* species. *Nucleic Acids Res*. **21:**4039–4045.

114. **Padmanabhan, S., J. Thakur, R. Siddharthan, and K. Sanyal.** 2008. Rapid evolution of Cse4p-rich centromeric DNA sequences in closely related pathogenic yeasts, *Candida albicans* and *Candida dubliniensis*. *Proc. Natl. Acad. Sci. USA* **105:**19797–19802.
115. **Perry, J., N. Kleckner, and G. V. Borner.** 2005. Bioinformatic analyses implicate the collaborating meiotic crossover/chiasma proteins Zip2, Zip3, and Spo22/Zip4 in ubiquitin labeling. *Proc. Natl. Acad. Sci. USA* **102:**17594–17599.
116. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
117. **Phan, Q. T., C. L. Myers, Y. Fu, D. C. Sheppard, M. R. Yeaman, W. H. Welch, A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol.* **5:**e64.
118. **Pujol, C., K. J. Daniels, S. R. Lockhart, T. Srikantha, J. B. Radke, J. Geiger, and D. R. Soll.** 2004. The closely related species *Candida albicans* and *Candida dubliniensis* can mate. *Eukaryot. Cell* **3:**1015–1027.
119. **Qiao, F., and T. R. Cech.** 2008. Triple-helix structure in telomerase RNA contributes to catalysis. *Nat. Struct. Mol. Biol.* **15:**634–640.
120. **Recca, J., and E. Mrak.** 1952. Yeasts occuring in citrus products. *Food Technol.* **6:**450–454.
121. **Reedy, J. L., A. M. Floyd, and J. Heitman.** 2009. Mechanistic plasticity of sexual reproduction and meiosis in the *Candida* pathogenic species complex. *Curr. Biol.* **19:**891–899.
122. **Reuss, O., and J. Morschhauser.** 2006. A family of oligopeptide transporters is required for growth of *Candida albicans* on proteins. *Mol. Microbiol.* **60:**795–812.
123. **Rodrigues de Miranda, L.** 1979. *Clavispora*, a new yeast genus of the Saccharomycetales. *Antonie van Leeuwenhoek* **45:**479–483.
124. **Rolland, T., C. Neuveglise, C. Sacerdot, and B. Dujon.** 2009. Insertion of horizontally transferred genes within conserved syntenic regions of yeast genomes. *PLoS One* **4:**e6515.
125. **Rossignol, T., C. Ding, A. Guida, C. d'Enfert, D. G. Higgins, and G. Butler.** 2009. Correlation between biofilm formation and the hypoxic response in *Candida parapsilosis*. *Eukaryot. Cell* **8:**550–559.
126. **Rossignol, T., P. Lechat, C. Cuomo, Q. Zeng, I. Moszer, and C. d'Enfert.** 2008. CandidaDB: a multi-genome database for *Candida* species and related Saccharomycotina. *Nucleic Acids Res.* **36:**D557–D561.
127. **Sadhu, C., M. J. McEachern, E. P. Rustchenko-Bulgac, J. Schmid, D. R. Soll, and J. B. Hicks.** 1991. Telomeric and dispersed repeat sequences in *Candida* yeasts and their use in strain identification. *J. Bacteriol.* **173:**842–850.
128. **Santos, M. A., and M. F. Tuite.** 1995. The CUG codon is decoded in vivo as serine and not leucine in *Candida albicans*. *Nucleic Acids Res.* **23:**1481–1486.
129. **Sanyal, K., M. Baum, and J. Carbon.** 2004. Centromeric DNA sequences in the pathogenic yeast *Candida albicans* are all different and unique. *Proc. Natl. Acad. Sci. USA* **101:**11374–11379.
130. **Schaller, M., C. Borelli, H. C. Korting, and B. Hube.** 2005. Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48:**365–377.
131. **Schurko, A. M., and J. M. Logsdon, Jr.** 2008. Using a meiosis detection toolkit to investigate ancient asexual "scandals" and the evolution of sex. *Bioessays* **30:**579–589.
132. **Schurko, A. M., J. M. Logsdon, Jr., and B. D. Eads.** 2009. Meiosis genes in *Daphnia pulex* and the role of parthenogenesis in genome evolution. *BMC Evol. Biol.* **9:**78.
133. **Schurko, A. M., M. Neiman, and J. M. Logsdon, Jr.** 2009. Signs of sex: what we know and how we know it. *Trends Ecol. Evol.* **24:**208–217.
134. **Shefer, K., Y. Brown, V. Gorkovoy, T. Nussbaum, N. B. Ulyanov, and Y. Tzfati.** 2007. A triple helix within a pseudoknot is a conserved and essential element of telomerase RNA. *Mol. Cell. Biol.* **27:**2130–2143.
135. **Sherman, D. J., T. Martin, M. Nikolski, C. Cayla, J. L. Souciet, and P. Durrens.** 2009. Génolevures: protein families and synteny among complete hemiascomycetous yeast proteomes and genomes. *Nucleic Acids Res.* **37:**D550–554.
136. **Silva, R. M., J. A. Paredes, G. R. Moura, B. Manadas, T. Lima-Costa, R. Rocha, I. Miranda, A. C. Gomes, M. J. Koerkamp, M. Perrot, F. C. Holstege, H. Boucherie, and M. A. Santos.** 2007. Critical roles for a genetic code alteration in the evolution of the genus *Candida*. *EMBO J.* **26:**4555–4565.
137. **Skrzypek, M. S., M. B. Arnaud, M. C. Costanzo, D. O. Inglis, P. Shah, G. Binkley, S. R. Miyasato, and G. Sherlock.** 2010. New tools at the Candida Genome Database: biochemical pathways and full-text literature search. *Nucleic Acids Res.* **38:**D428–D432.
138. **Slutsky, B., M. Staebell, J. Anderson, L. Risen, M. Pfaller, and D. R. Soll.** 1987. "White-opaque transition": a second high-frequency switching system in *Candida albicans*. *J. Bacteriol.* **169:**189–197.
139. **Soll, D. R.** 2008. *Candida* biofilms: is adhesion sexy? *Curr. Biol.* **18:**R717–R720.
140. **Srikantha, T., S. A. Lachke, and D. R. Soll.** 2003. Three mating type-like loci in *Candida glabrata*. *Eukaryot. Cell* **2:**328–340.
141. **Stanhope, M. J., A. Lupas, M. J. Italia, K. K. Koretke, C. Volker, and J. R. Brown.** 2001. Phylogenetic analyses do not support horizontal gene transfers from bacteria to vertebrates. *Nature* **411:**940–944.
142. **Stehr, F., A. Felk, A. Gacser, M. Kretschmar, B. Mahnss, K. Neuber, B. Hube, and W. Schafer.** 2004. Expression analysis of the *Candida albicans* lipase gene family during experimental infections and in patient samples. *FEMS Yeast Res.* **4:**401–408.
143. **Stokes, C., G. P. Moran, M. J. Spiering, G. T. Cole, D. C. Coleman, and D. J. Sullivan.** 2007. Lower filamentation rates of *Candida dubliniensis* contribute to its lower virulence in comparison with *Candida albicans*. *Fungal Genet. Biol.* **44:**920–931.
144. **Suh, S. O., C. J. Marshall, J. V. McHugh, and M. Blackwell.** 2003. Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts. *Mol. Ecol.* **12:**3137–3145.
145. **Syvanen, M.** 1985. Cross-species gene transfer; implications for a new theory of evolution. *J. Theor. Biol.* **112:**333–343.
146. **Tavanti, A., A. D. Davidson, M. J. Fordyce, N. A. Gow, M. C. Maiden, and F. C. Odds.** 2005. Population structure and properties of *Candida albicans*, as determined by multilocus sequence typing. *J. Clin. Microbiol.* **43:**5601–5613.
147. **Tavanti, A., A. D. Davidson, N. A. Gow, M. C. Maiden, and F. C. Odds.** 2005. *Candida orthopsilosis* and *Candida metapsilos*is spp. nov. to replace *Candida parapsilosis* groups II and III. *J. Clin. Microbiol.* **43:**284–292.
148. **Taylor, D. J., and J. Bruenn.** 2009. The evolution of novel fungal genes from non-retroviral RNA viruses. *BMC Biol.* **7:**88.
149. **Tsong, A. E., M. G. Miller, R. M. Raisner, and A. D. Johnson.** 2003. Evolution of a combinatorial

transcriptional circuit: a case study in yeasts. *Cell* **115:**389–399.

150. **Tsong, A. E., B. B. Tuch, H. Li, and A. D. Johnson.** 2006. Evolution of alternative transcriptional circuits with identical logic. *Nature* **443:**415–420.
151. **Tzung, K. W., R. M. Williams, S. Scherer, N. Federspiel, T. Jones, N. Hansen, V. Bivolarevic, L. Huizar, C. Komp, R. Surzycki, R. Tamse, R. W. Davis, and N. Agabian.** 2001. Genomic evidence for a complete sexual cycle in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **98:**3249–3253.
152. **Ulyanov, N. B., K. Shefer, T. L. James, and Y. Tzfati.** 2007. Pseudoknot structures with conserved base triples in telomerase RNAs of ciliates. *Nucleic Acids Res.* **35:**6150–6160.
153. **van der Walt, J. P.** 1966. *Lodderomyces*, a new genus of the Saccharomycetaceae. *Antonie van Leeuwenhoek* **32:**1–5.
154. **van der Walt, J. P., M. B. Taylor, and N. V. Liebenberg.** 1977. Ploidy, ascus formation and recombination in *Torulaspora* (*Debaryomyces*) *hansenii*. *Antonie van Leeuwenhoek* **43:**205–218.
155. **van het Hoog, M., T. J. Rast, M. Martchenko, S. Grindle, D. Dignard, H. Hogues, C. Cuomo, M. Berriman, S. Scherer, B. B. Magee, M. Whiteway, H. Chibana, A. Nantel, and P. T. Magee.** 2007. Assembly of the *Candida albicans* genome into sixteen supercontigs aligned on the eight chromosomes. *Genome Biol.* **8:**R52.
156. **Verstrepen, K. J., A. Jansen, F. Lewitter, and G. R. Fink.** 2005. Intragenic tandem repeats generate functional variability. *Nat. Genet.* **37:**986–990.
157. **Vilela, M. M., K. Kamei, A. Sano, R. Tanaka, J. Uno, I. Takahashi, J. Ito, K. Yarita, and M. Miyaji.** 2002. Pathogenicity and virulence of *Candida dubliniensis*: comparison with *C. albicans*. *Med. Mycol.* **40:**249–257.
158. **Wang, H., Z. Xu, L. Gao, and B. Hao.** 2009. A fungal phylogeny based on 82 complete genomes using the composition vector method. *BMC Evol. Biol.* **9:**195.
159. **Wickerham, L. J., and K. A. Burton.** 1954. A clarification of the relationship of *Candida guilliermondii* to other yeasts by a study of their mating types. *J. Bacteriol.* **68:**594–597.
160. **Wingard, J. R.** 1995. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. *Clin. Infect. Dis.* **20:**115–125.
161. **Wong, S., M. A. Fares, W. Zimmermann, G. Butler, and K. H. Wolfe.** 2003. Evidence from comparative genomics for a complete sexual cycle in the 'asexual' pathogenic yeast *Candida glabrata*. *Genome Biol.* **4:**R10.
162. **Wood, V., R. Gwilliam, M. A. Rajandream, M. Lyne, R. Lyne, A. Stewart, J. Sgouros, N. Peat, J. Hayles, S. Baker, D. Basham, S. Bowman, K. Brooks, D. Brown, S. Brown, T. Chillingworth, C. Churcher, M. Collins, R. Connor, A. Cronin, P. Davis, T. Feltwell, A. Fraser, S. Gentles, A. Goble, N. Hamlin, D. Harris, J. Hidalgo, G. Hodgson, S. Holroyd, T. Hornsby, S. Howarth, E. J. Huckle, S. Hunt, K. Jagels, K. James, L. Jones, M. Jones, S. Leather, S. McDonald, J. McLean, P. Mooney, S. Moule, K. Mungall, L. Murphy, D. Niblett, C. Odell, K. Oliver, S. O'Neil, D. Pearson, M. A. Quail, E. Rabbinowitsch, K. Rutherford, S. Rutter, D. Saunders, K. Seeger, S. Sharp, J. Skelton, M. Simmonds, R. Squares, S. Squares, K. Stevens, K. Taylor, R. G. Taylor, A. Tivey, S. Walsh, T. Warren, S. Whitehead, J. Woodward, G. Volckaert, R. Aert, J. Robben, B. Grymonprez, I. Weltjens, E. Vanstreels, M. Rieger, M. Schafer, S. Muller-Auer, C. Gabel, M. Fuchs, A. Dusterhoft, C. Fritzc, E. Holzer, D. Moestl, H. Hilbert, K. Borzym, I. Langer, A. Beck, H. Lehrach, R. Reinhardt, T. M. Pohl, P. Eger, W. Zimmermann, H. Wedler, R. Wambutt, B. Purnelle, A. Goffeau, E. Cadieu, S. Dreano, S. Gloux, et al.** 2002. The genome sequence of *Schizosaccharomyces pombe*. *Nature* **415:**871–880.
163. **Wright, S., and D. Finnegan.** 2001. Genome evolution: sex and the transposable element. *Curr. Biol.* **11:**R296–R299.
164. **Yi, S., N. Sahni, C. Pujol, K. J. Daniels, T. Srikantha, N. Ma, and D. R. Soll.** 2009. A *Candida albicans*-specific region of the alpha-pheromone receptor plays a selective role in the white cell pheromone response. *Mol. Microbiol.* **71:**925–947.
165. **Yu, X., and A. Gabriel.** 2004. Reciprocal translocations in *Saccharomyces cerevisiae* formed by nonhomologous end joining. *Genetics* **166:**741–751.
166. **Zhao, X., K. J. Daniels, S. H. Oh, C. B. Green, K. M. Yeater, D. R. Soll, and L. L. Hoyer.** 2006. *Candida albicans* Als3p is required for wild-type biofilm formation on silicone elastomer surfaces. *Microbiology* **152:**2287–2299.
167. **Zhao, X., S. H. Oh, G. Cheng, C. B. Green, J. A. Nuessen, K. Yeater, R. P. Leng, A. J. Brown, and L. L. Hoyer.** 2004. *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. *Microbiology* **150:**2415–2428.
168. **Zhao, X., S. H. Oh, and L. L. Hoyer.** 2007. Deletion of *ALS5*, *ALS6* or *ALS7* increases adhesion of *Candida albicans* to human vascular endothelial and buccal epithelial cells. *Med. Mycol.* **45:**429–434.
169. **Zhao, X., S. H. Oh, K. M. Yeater, and L. L. Hoyer.** 2005. Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* **151:** 1619–1630.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch4

4

The Genetic Code of the *Candida* CTG Clade

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Living systems have evolved sophisticated mechanisms to ensure highly accurate DNA replication, transcription, and translation. Indeed, global DNA replication error ranges from 10^{-10} to 10^{-11}, while the transcriptional error rate is on the order of 10^{-6} (6) and translational error rates in bacteria and yeasts are on the order of 10^{-4} and 10^{-5}, respectively (52). Therefore, despite being tightly regulated and highly conserved among the three kingdoms of life, translation is the least accurate of the three biological information processes.

The translational machinery is composed of a large number of different molecules, namely, mRNAs, tRNAs, amino acids, translational factors, ribosomes, and aminoacyl-tRNA synthetases (aaRSs), and there are key steps where the accuracy of the genetic code decoding is controlled. These include the aminoacylation of tRNAs by aaRSs, discrimination of correctly charged tRNAs by the elongation factors (EF1A), and the codon-anticodon pairing at the A-site of the ribosome. Despite this, translational errors arise from both incorrect aminoacylation and codon mispairing with average frequencies of 10^{-4} to 10^{-5} (28, 33). Interestingly, hyperaccurate ribosomes slow down the growth, indicating that protein synthesis accuracy is a compromise between decoding fidelity and speed (33). Missense errors are the most frequent translational errors under physiological conditions (19) and are normally deleterious; however, a certain percentage of such errors are neutral or semineutral. This is related to biases in the genetic code structure that maximize errors with chemically similar amino acids (18, 41, 45).

The genetic code was thought to be universal and frozen, but several deviations to its standard structure have been discovered in prokaryotic, eukaryotic, and organellar translation systems over the last 30 years (reviewed in reference 18), and it is now clear that some of these alterations evolved gradually through codon decoding ambiguity (18, 41, 45). Most alterations and expansions of the genetic code are mediated through structural changes in the protein synthesis machinery, in particular in tRNAs, aaRSs, and elongation and termination factors (41, 42, 58). In this chapter, we discuss the most recent findings on the reassignment mechanism of CUG codons from leucine to serine in various *Candida* and non-*Candida* species, the so-called CTG clade (3). We highlight how the *Candida albicans* model system is furthering our understanding of the evolution of the genetic code, and we explain how this genetic code alteration shaped the biology of the CTG clade species.

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OVERVIEW OF THE EVOLUTIONARY MECHANISMS OF CODON REASSIGNMENT

Codon reassignments show that the genetic code evolves even in organisms with complex genomes and proteomes (41). However, the accumulation of genetic code alterations in mitochondria, whose genomes encode a minimal number of proteins (18), suggests that genome size and complexity restrict evolution of the genetic code. Nevertheless, the diversity and phylogenetic analysis of genetic code alterations show that they occur in distinct phylogenetic lineages and evolve from the standard genetic code rather than from alternative and ancient codes. Interestingly, codons starting with A or U often change their identity, while codons starting with G never alter their identity (18). There are only two cases where C-starting codons changed identity, namely, the reassignment of CUN codons from leucine to threonine in yeast mitochondria (21) and reassignment of the CUG codon from leucine to serine in various *Candida* species (43). These findings indicate that the strength of the first codon-anticodon base pair limits codon identity alterations and supports the hypothesis that codon decoding efficiency is a key factor in the evolution of genetic code alterations (26). Finally, certain codons are rather unstable, as they changed identity more than once. For example, the arginine AGG codons changed identity to Ser, Gly, and STOP (reviewed in reference 18), and STOP codons changed their identity to different amino acids, namely, Trp, Tyr, Glu, Gln, and Cys in various organisms (31). The theories discussed below explain the evolution of genetic code alterations.

The codon capture theory postulates that genetic code changes result from genome G+C biases on codon usage (30). Biased G+C pressure modulates the usage frequency of

the third nucleotide position of codons ($N_1N_2N_3$) and does not alter protein amino acid sequences due to the degeneracy of the genetic code, allowing for neutral evolution of genetic code alterations. This theory predicts that under strong G+C bias some codons may disappear from genomes (30) and is supported by the disappearance of the arginine CGG codon in *Mycoplasma capricolum*, whose genome has 25% G+C. In this case, the arginine CGG codon is unassigned and its cognate $tRNA^{Arg}_{CCG}$ gene also vanished from the genome (27). Conversely, in the genome of *Micrococcus luteus*, where A+T content is 26% only, the A/T-rich codons UUA, AUA, and AGA were erased (15, 29). Similar genome biases explain the evolution of genetic code changes in the A+T-rich genomes of mitochondria (31). The theory also postulates that unassigned and rarely used codons are primary targets for reassignment because their capture by mutant misreading tRNAs has neutral or minimal impact on the proteome, i.e., does not decrease fitness.

The ambiguous-intermediate theory postulates that genetic code alterations are driven by selection and result from mutations in components of the translational machinery (45, 46). In particular, mutant tRNAs with altered codon decoding properties are important driving forces of genetic code alterations and mutations that alter release factors; tRNA-modifying enzymes and aaRSs also have potential to create codon ambiguity and promote codon reassignment (46). This hypothesis postulates that codon ambiguity creates a transition stage during which a codon is decoded by its cognate tRNA and by a mutant tRNA belonging to a different amino acid family. The mutant misreading tRNA gradually displaces the wild-type cognate tRNA, taking over the decoding of the ambiguous codon. This theory does not require codon disappearance prior to reassignment and implies that codon ambiguity provides some sort of selective advantage to drive codon reassignment to completion (45, 46). There are several examples of ambiguous translation that support this model: (i) in *Bacillus subtilis* the UGA stop codon is recognized by both release factor 2 and a tryptophan suppressor tRNA (22, 24); (ii) in *Schizosaccharomyces pombe* the mutants *scn1* and *scn2* translate the Thr codons ACA as Ala due to a single point mutation in the anticodon of $tRNA^{Ala}_{UGC}$ (UGC →UGU) (17); (iii) in *Arabidopsis thaliana* auxin-resistant mutants exhibit a mutation in $tRNA^{Ala}_{CGC}$ (CGC →CAC), allowing translation of Val GUG as Ala (35); (iv) in the ciliate *Euplotes crassus* the codon UGA specifies selenocysteine and cysteine, and interestingly, this dual codon assignment can occur within the same gene (56). Surprisingly, these codon ambiguities have little or no visible impact on growth rate. An additional example is *C. albicans* and other fungal species of the CTG clade, where Leu CUG codons are decoded as both Ser and Leu via a single ambiguous $tRNA^{Ser}_{CAG}$ (40, 43). It is not yet clear how codon ambiguity creates selective advantages because normally it reduces growth rate. However, recent studies show that cells are highly tolerant to codon ambiguity and that it may be advantageous under stress. For example, *E. coli* can survive with 10% of misacylated tRNA (38) and *C. albicans* can cope with 28% of CUG ambiguity (11). In other words, there is significant flexibility in the genetic code to support codon reassignment.

Despite the differences between those two theories, they are not mutually exclusive. Sengupta and Higgs proposed a unifying model for codon identity changes, the so-called gain-loss theory (47). This theory is based on the observation that codon reassignments always involve both a gain and a loss. The authors consider gain the new tRNA for the reassigned codon or a gain of function of an existing tRNA (due to a mutation or a base modification) and loss the deletion of tRNA or release factor genes or loss of function of such genes, again, due to a mutation or a base modification. According to this model, the codon capture theory and the ambiguous-intermediate theory act synergistically, as it is the strength and the frequency of the loss or the gain that determines which mechanism is favored. For example, if a codon identity change requires a new modified base, a loss seems simpler than a gain because it is easier to lose a tRNA gene than to gain a novel enzyme to create such modification; hence, the codon capture model would be favored (47).

CUG REASSIGNMENT IN THE CTG CLADE

The *Candida* genus contains several species that decode the leucine CUG codon as serine. Kawaguchi and colleagues were the first to describe a nuclear sense-to-sense codon reassignment in a eukaryote, namely, in *Candida cylindracea*, where the Leu CUG codon was decoded as Ser (16). The first studies using in vitro translation assays demonstrated that a tRNA species mediated a nonstandard mRNA decoding event (55), which was subsequently confirmed as the nonstandard decoding of Leu CUG codons as Ser (43). Subsequent studies showed that the CUG codon was ambiguously decoded as both Ser (major) and Leu (minor) in all but one of the CTG clade species (11, 54), namely, in *C. cylindracea*.

To date, the reassignment of the CUG codon from Leu to Ser has been described to occur in 75 *Candida* species and in other closely related yeasts (53), namely, *Pichia stipitis*, *Debaryomyces hansenii*, and *Lodderomyces elongisporus* (9). Interestingly, CUG decoding in the *Candida* genus is heterogeneous (see chapters 2 and 3) (53), indicating that reassignment of the CUG codon followed different routes among the different species. Some *Candida* species translate the CUG codon exclusively as Leu, namely, *C. glabrata* and *C. krusei*, while *C. cylindracea* decodes it as Ser only. In most species, namely, in *C. zeylanoides*, *C. dubliniensis*, and *C. albicans*, the CUG codon is ambiguous, meaning that it is simultaneously translated as Leu and Ser.

THE UNIQUE *CANDIDA* $tRNA^{Ser}_{CAG}$

The change of identity of the CUG codon in the CTG clade is mediated by a novel $tRNA^{Ser}_{CAG}$ (Fig. 1). This tRNA is a hybrid molecule containing an anticodon stem similar to those of Leu tRNAs, while the remaining part of its structure is similar to that of Ser tRNAs (34, 42, 54). This allows for recognition of this mutant tRNA by both seryl-tRNA synthetases and leucyl-tRNA synthetases (LeuRS), and consequently, it is charged with Ser and Leu. Therefore, most of the CTG clade species contain in their cytoplasm two isoforms of $tRNA^{Ser}_{CAG}$, namely, ser-$tRNA^{Ser}_{CAG}$ and leu-$tRNA^{Ser}_{CAG}$, which compete with each other for CUG decoding at the A-site of the ribosome during translation (11, 39, 54). The mischarged leu-$tRNA^{Ser}_{CAG}$ is neither edited by the LeuRS nor discriminated by the translation factor eukaryotic elongation factor 1A (eEF1A), and consequently, the CUG codon is ambiguously decoded as Ser and Leu in vivo. This in vivo ambiguity was quantified by mass spectrometry and under normal laboratory growth conditions; *C. albicans* inserts 97% of Ser and 3% of Leu at CUGs, but these values are apparently flexible, as Leu incorporation increases up to 5% in *C. albicans* grown at pH 4.0 (Fig. 2) (11).

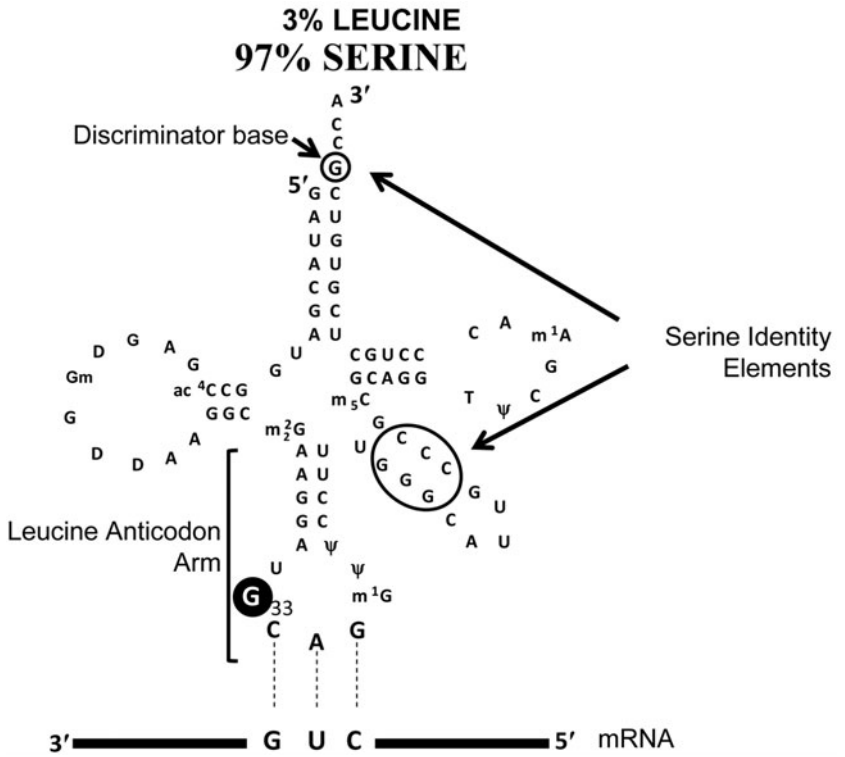


FIGURE 1 Secondary structure of $tRNA_{CAG}^{Ser}$. *C. albicans* $tRNA_{CAG}^{Ser}$ is a hybrid tRNA with identity elements for both leucyl- and seryl-tRNA synthetases. Its anticodon arm is characteristic of leucine tRNAs, whereas the acceptor stem and the variable arm are characteristic of serine tRNAs. G_{33} played a critical role in the reassignment of CUG codons from leucine to serine. The discriminator base (G_{73}) is characteristic of the serine family of tRNAs (40). 10.1128/9781555817176.ch4f1

From a structural point of view, the most striking feature of this $tRNA_{CAG}^{Ser}$ is the presence of guanosine at position 33 (G_{33}), immediately adjacent to the 5′ base of the 5′-CAG-3′ anticodon (Fig. 1). Some mitochondrial tRNAs contain C_{33}, but prokaryotic and eukaryotic elongation tRNAs have a highly conserved uridine at position 33 (U_{33})

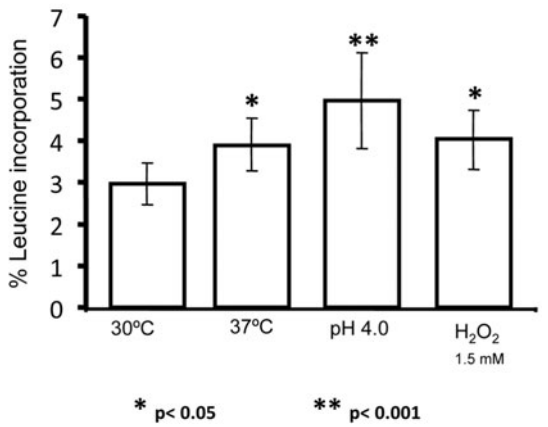


FIGURE 2 CUG ambiguity is sensitive to environmental cues. Leucine incorporation at CUG codons in vivo is sensitive to environmental conditions, namely, temperature, low pH, and oxidative stress. Adapted from reference 11. *, $P < 0.05$; **, $P < 0.001$. 10.1128/9781555817176.ch4f2

(51) which is required for the correct turn of the phosphate backbone (U-turn) and stacking of the anticodon bases (20, 57). G_{33} induces a long-range distortion of the top of the anticodon stem of the tRNA which makes it less susceptible to V1 ribonuclease, an enzyme that specifically targets helical or stacked bases in RNAs (34). The G_{33} mutation had an important role in CUG reassignment (42, 44, 54), as it lowered the leucylation levels of the tRNA and decreased the decoding efficiency of the $tRNA_{CAG}^{Ser}$. In other words, it decreased the toxicity of the mutant $tRNA_{CAG}^{Ser}$ (34, 42). Finally, the discriminator base of the $tRNA_{CAG}^{Ser}$ is guanosine (G_{73}), which is an identity element for the serine tRNA family, and it is not yet clear how the LeuRS recognizes a tRNA with G_{73}. In *Saccharomyces cerevisiae* a single change of A_{73} to G_{73} of a $tRNA^{Leu}$ is sufficient to convert its identity from leucine to serine (50), suggesting that the *C. albicans* LeuRS should not recognize the $tRNA_{CAG}^{Ser}$. But, the $tRNA_{CAG}^{Ser}$ contains A_{35} and m^1G_{37} in the anticodon loop, which are recognized by the LeuRS (Fig. 1) (50), and this may explain the recognition of the $tRNA_{CAG}^{Ser}$ by the LeuRS.

THE REASSIGNMENT OF CUG CODONS IN THE CTG CLADE

The CTG clade codon reassignment strongly supports the ambiguous-intermediate theory, as CUG reassignment is mediated by an ambiguous tRNA which introduces Ser and Leu at CUG positions (42, 43, 54). The ambiguous CUG

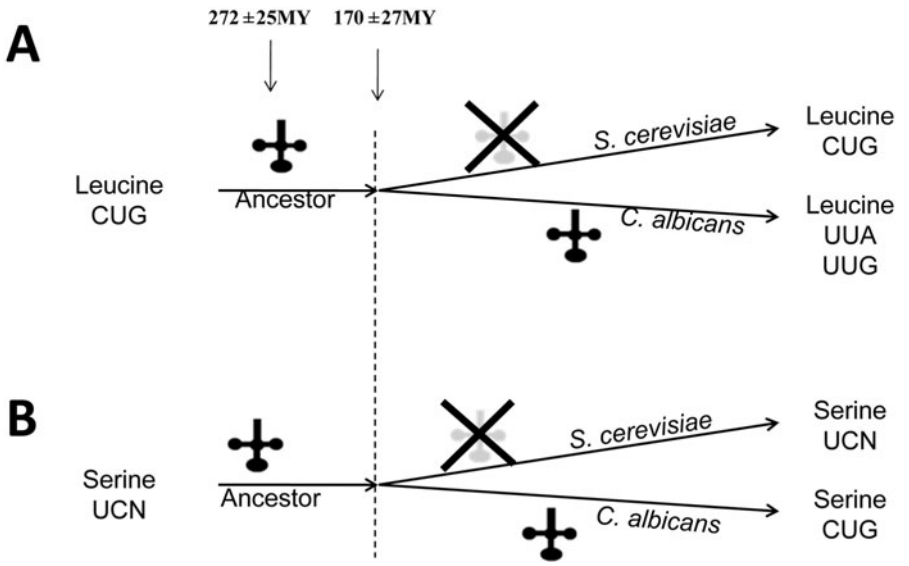


FIGURE 3 Evolution of CUG codons in the CTG clade. The redefinition of the identity of the CUG codon from leucine to serine in the CTG clade started with the appearance of a novel serine $tRNA_{CAG}^{Ser}$ 272 ± 25 million years ago and evolved gradually. The *S. cerevisiae* and the *C. albicans* lineages diverged 170 ± 27 million years ago, and $tRNA_{CAG}^{Ser}$ was maintained in the CTG clade lineage (altered genetic code) but was lost in the *S. cerevisiae* lineage (standard genetic code). The novel serine $tRNA_{CAG}^{Ser}$ forced ancestor CUG codons to mutate to UUG and UUA leucine codons (A). Simultaneously, newCUG codons appeared via mutation of UCN serine codons (B). Adapted from reference 23. 10.1128/9781555817176.ch4f3

codon is a rarely used codon, supporting the above-mentioned hypothesis that the codon usage is critical for reassignment. Interestingly, the novel $tRNA_{CAG}^{Ser}$ captured many "new" CUG codons via mutation from codons coding for Ser or amino acids with similar chemical properties (3, 23), and consequently, the CUG codons of the CTG clade species appeared recently and are unrelated to the Leu CUG codons present in the non-CTG clade species.

Comparative genomics and molecular phylogeny studies have shown that $tRNA_{CAG}^{Ser}$ appeared 272 ± 25 million years ago—prior to the divergence between the *Saccharomyces* and *Candida* genera, which is dated at 170 ± 27 million years ago (Fig. 3) (23). $tRNA_{CAG}^{Ser}$ appeared via an insertion of an adenosine in the anticodon of a serine $tRNA_{CGA}^{Ser}$ gene; however, the full reconstruction of the CUG identity alteration pathway has not yet been carried out, and the complete sequence of events involved in CUG reassignment is not yet understood.

The appearance of the $tRNA_{CAG}^{Ser}$ created a unique situation where CUG codons became decoded by two distinct tRNA species: the new mutant ser-$tRNA_{CAG}^{Ser}$ and the old cognate leu-$tRNA_{CAG}^{Leu}$. Those two tRNAs competed for approximately 100×10^6 years for CUG codons and introduced significant ambiguity at CUG positions (23, 59). $tRNA_{CAG}^{Ser}$ was lost in the ancestral lineage of *Saccharomyces* spp. and these species reverted CUG identity to its original meaning, while the ancestors of *Candida* spp. lost the old $tRNA_{CAG}^{Leu}$ and retained the mutant $tRNA_{CAG}^{Ser}$, thus changing the identity of CUG codons (23).

CUG ambiguity imposed strong negative pressure on CUG codon usage and triggered a massive mutational change of ancestral CUG codons to UUG or UUA Leu codons. Indeed, 98% of the CUG codons of the *Candida* ancestor were erased from its genome. Simultaneously, $tRNA_{CAG}^{Ser}$ captured new CUG codons from the serine UCN codon family (Fig. 3) (23).

DECODING OF CUN CODONS IN *CANDIDA* SPP

The reassignment of the CUG codon from Leu to Ser also had a strong impact on the decoding of CUN codons (26). In the CTG clade species, CUG codons are decoded by $tRNA_{CAG}^{Ser}$ and CUA, CUU, and CUC codons are decoded by a single tRNA with a 5′-IAG-3′ anticodon ($tRNA_{IAG}^{Leu}$) (Fig. 4). The decoding of three different codons by a single $tRNA_{IAG}^{Leu}$ is possible because the inosine (I) present at the first position of the anticodon decodes codons ending in A, C, or U through extended wobble interactions, but the strength of decoding is variable. The CUU codon is cognate for this tRNA and interacts strongly with it. A similar situation occurs for the 5′-IAG-3′ interaction with the CUC codon; however, the 5′-IAG-3′-CUA interaction is weak and reduced CUA usage (23). Conversely, in *S. cerevisiae* the CUN codon family is decoded by two tRNAs, namely, $tRNA_{UAG}^{Leu}$, which decodes CUA and CUG codons, and $tRNA_{GAG}^{Leu}$, which decodes CUC and CUU codons. In *S. pombe* the CUN codon family is decoded by three different tRNAs, a $tRNA_{GAG}^{Leu}$ which decodes CUC and CUU codons, a $tRNA_{UAG}^{Leu}$ which decodes CUA, and a $tRNA_{CAG}^{Leu}$ which decodes CUG codons. Therefore, the appearance of the novel $tRNA_{CAG}^{Ser}$ influenced both the CUN decoding in *C. albicans* and *S. cerevisiae* and the evolution of leucine tRNAs in the CTG clade species (3, 26).

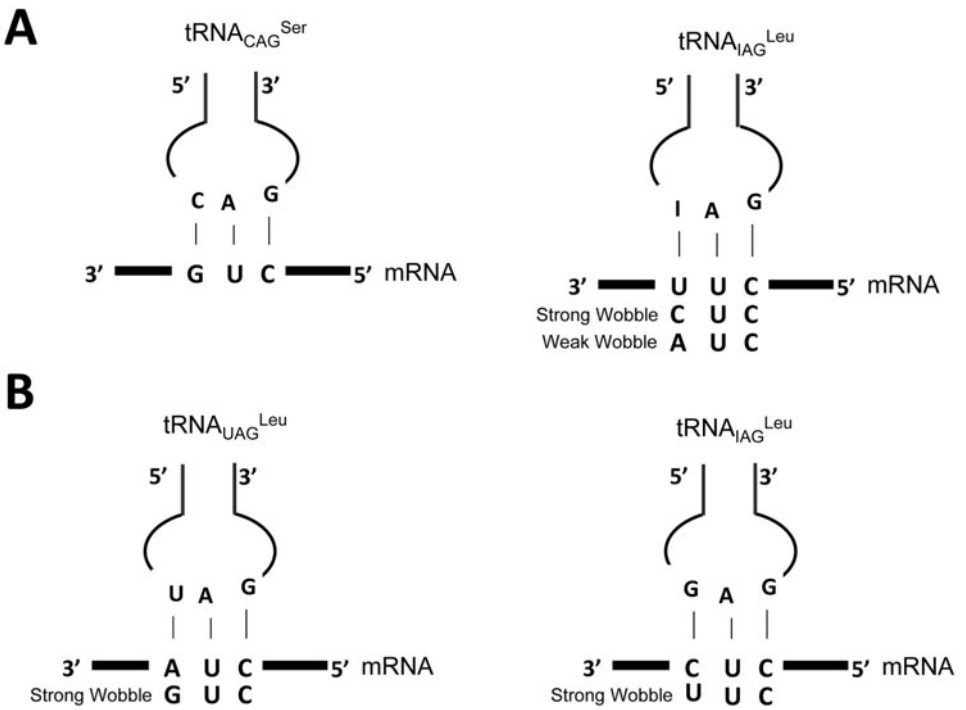


FIGURE 4 Decoding of CUN codon family in *C. albicans* and *S. cerevisiae*. (A) *C. albicans* decodes CUG codons using a cognate $tRNA_{CAG}^{Ser}$, while the CUU, CUC, and CUA codons are decoded by $tRNA_{IAG}^{Leu}$. (B) In *S. cerevisiae*, $tRNA_{UAG}^{Leu}$ decodes CUA and CUG codons and $tRNA_{GAG}^{Leu}$ decodes the CUU and CUC codons. 10.1128/9781555817176.ch4f4

THE IMPACT OF CUG AMBIGUITY ON *C. ALBICANS* BIOLOGY

C. albicans mistranslates CUG constitutively and tolerates Leu misincorporation at CUG positions at a very high level—from 0.6% up to 4.9%. Considering that the basal mRNA decoding error in yeasts is on the order of 10^{-5} (52), such a Leu misincorporation rate represents between a 600- and 4,900-fold increase in decoding error. Recent studies showed that *C. albicans* tolerates up to 28% of Leu incorporation at CUG codons, which represents a 28,000-fold increase in typical translational error (11). This results in genome instability, activation of morphogenesis, and a number of other phenotypes that range from lipase to proteinase production to flocculation (Fig. 5) (25). These highly ambiguous cells generate cell population and colony morphology heterogeneity, aerial hyphae, and white-opaque sectoring, with colonies often forming long filaments (25). Interestingly, morphological variation, growth at high temperature, and yeast-hypha transition, as well as proteinase and lipase secretion and expression of adhesins, play important roles in *Candida* infection (2, 4). The phenotypic diversity induced by CUG ambiguity exposes some of these virulence traits, suggesting that CUG ambiguity may be relevant to pathogenesis (25) and that *C. albicans* may have evolved unique mechanisms to take advantage of its genetic code alteration.

THE STATISTICAL PROTEOME OF *C. ALBICANS*

The double identity of the CUG codon implies that both Ser and Leu are inserted at CUG positions on a genome-wide scale. Therefore, each *C. albicans* protein is represented by a mixture of molecules containing Leu or Ser at CUG positions, which increases proteome complexity rather dramatically.

In order to comprehend the global impact of such an ambiguous decoding event, one has to analyze the CUG distribution in the *C. albicans* genome. This organism has 13,074 CUG codons (haploid genome) distributed over 66% of its genes at a frequency of 1 to 38 CUGs per gene, although most of the genes have between one and five CUG codons (57.7%) (Fig. 6A). Considering that two different amino acids can be inserted at CUG positions, the number of protein molecules increases exponentially with the increasing number of CUG codons per gene (2^n). Indeed, the 6,438 protein-encoding genes of *C. albicans* have the potential to produce 283 billion different protein molecules (Fig. 6B) (11).

Since each gene is translated as an array of protein molecules containing Leu or Ser at CUG positions, the *C. albicans* proteome has a statistical nature (11). In other words, each cell has a unique combination of proteins and the probability of finding two identical cells in a population is extremely small, even if they are expressing the same genes. For instance, in a cell with 3% of Leu incorporation at the CUG codon, the probability of a protein encoded by a gene with three CUGs to contain one Leu is 8.36%, whereas in a cell with 28% of Leu incorporation that probability is 43% (Fig. 7). If one takes into consideration the number of molecules per cell for each protein, a picture of extreme proteome complexity emerges. Indeed, in *S. cerevisiae*, low- and high-abundance proteins are represented by 50 and 10^6 molecules per cell, respectively (10). Assuming that (i) all

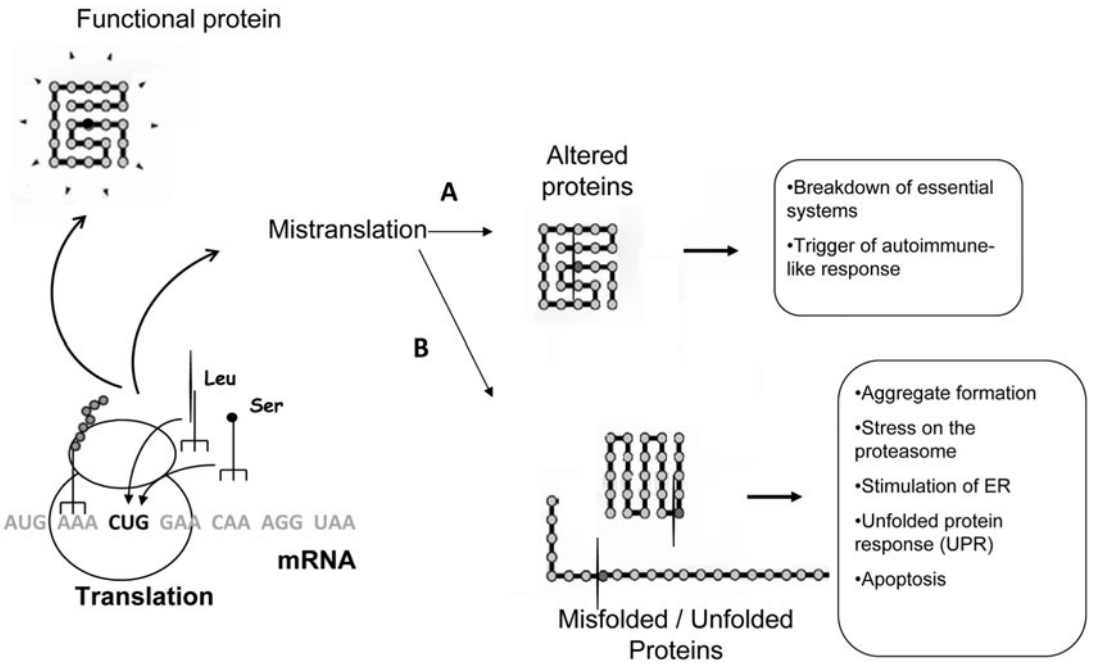
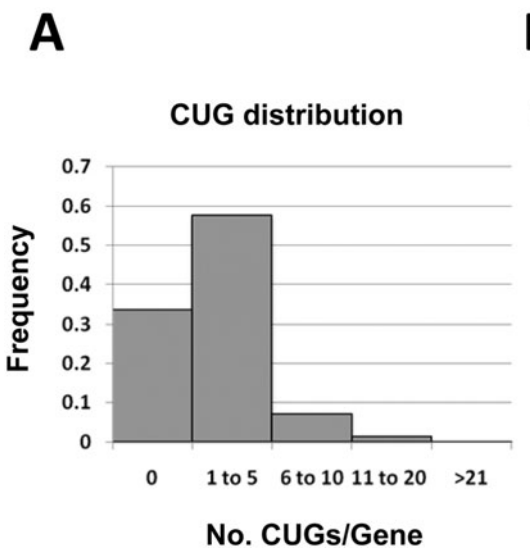


FIGURE 5 General consequences of CUG ambiguity. Mistranslation results in formation of either mutant proteins that can still fold (A) or misfolded and unfolded proteins which impose a burden on cell physiology and proteome homeostasis (B). 10.1128/9781555817176.ch4f5

C. albicans genes are expressed, (ii) 10% of the proteins with the lowest codon adaptation index (CAI) values are represented by 5,000 molecules/cell, (iii) 10% of the proteins with the highest CAI value are represented by 50,000 molecules/cell, and (iv) the remaining 80% of genes are represented by 20,000 molecules/cell (10), then the number of novel proteins encoded by *C. albicans* for CUG ambiguity levels of 3% is 6.7×10^6. This value increases up to 10.7×10^6 in cells grown at pH 4.0. The engineered *C. albicans* cells expressing 28% of CUG ambiguity are able to produce



B

No. of CUG per gene	No. Possible proteins	No. Genes with n CUG	Total different proteins arising
0	1	2,169	2,169
1	2	1,439	2,878
2	4	953	3,812
3	8	609	4,872
4	16	423	6,768
5	32	289	9,248
10	1,024	45	46,080
…	…	…	…
38	2.75E+11	1	2.7488E+11
	Total	6438	283×10^9

FIGURE 6 Distribution of CUG codons in *C. albicans* genes. (A) In *C. albicans* one-third of the genes do not have CUG codons. The majority (57.7%) contain between 1 and 5 CUG codons, and 7.1% have between 6 and 10. Only a small fraction of genes have more than 10 CUG codons. (B) Such CUG distribution and its ambiguous decoding expand the *C. albicans* proteome exponentially. The total theoretical number of combinatorial proteins encoded by the *C. albicans* genome is 283 billion (11). 10.1128/9781555817176.ch4f6

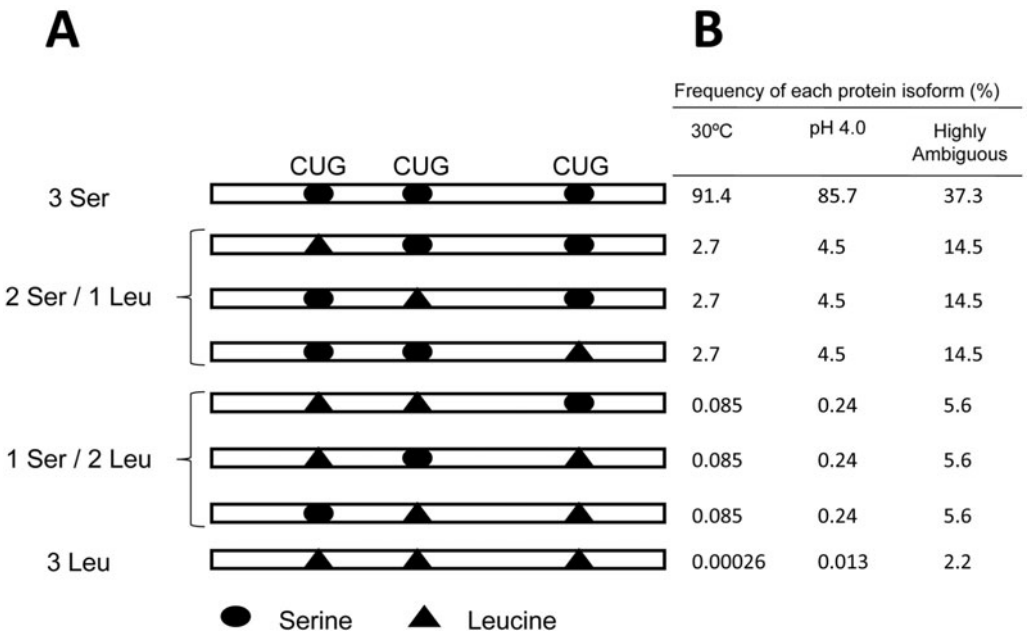


FIGURE 7 Production of combinatorial proteins in C. *albicans*. (A) The diagram shows the combinatorial proteins encoded by a gene containing three CUG codons. (B) The probability of synthesis of the protein isoforms depends on the level of leucine incorporation: 3% at 30ºC, 5% at pH 4.0, and 28% in recombinant cells. 10.1128/9781555817176.ch4f7

42.8×10^6 different protein molecules. Therefore, the C. *albicans* proteome is highly flexible and the number of different proteins is far higher than the number of genes. Whether this proteome flexibility is relevant for pathogenesis, adaptation, and evolution remains to be determined.

THE *CANDIDA ALBICANS* GENOME IS OPTIMIZED FOR CUG AMBIGUITY

C. *albicans* strongly represses CUG usage in genes with the highest expression levels (higher CAI values) and accumulates CUG codons in genes that are expressed at low levels. While 83% of the genes with the highest expression levels do not have CUG codons, 81% of the genes that are expressed at low levels have at least one CUG codon. This contrasts with CUG usage in S. *cerevisiae*, where only 56% of the highly expressed genes do not have CUG codons (Fig. 8) and one-third of the genes have more than five CUG codons (23).

The link between CUG distribution and protein expression levels becomes clearer if one calculates the number of mutant protein molecules that are synthesized in ambiguous S. *cerevisiae* and C. *albicans* cells. Expression of the C. *albicans* $tRNA_{CAG}^{Ser}$ in S. *cerevisiae* induces 1.4% misreading of CUG codons and decreases the growth rate by 47.9% (42, 49), while in C. *albicans* 3% of CUG misreading is physiological and its increase of up to 28% does not affect growth rate. In S. *cerevisiae*, 1.4% of serine misincorporation at CUGs generates 7.9 million mutant protein molecules, while 3% of leucine misincorporation at C. *albicans* CUG codons generates 6.3 million mutant protein molecules. These mistranslation levels induce the general stress response in S. *cerevisiae* but do not do so in C. *albicans* (8, 49), showing that the C. *albicans* genome minimizes CUG ambiguity.

THE USAGE OF CUG CODONS IN *C. ALBICANS*

In C. *albicans* the CUG codon is rarely used (0.43% usage) (Fig. 9). The specific codon usage (SCU) can be used to measure the relative frequency of CUG codons, as it normalizes the codon usage data in terms of Ser abundance (13) and is more informative than the absolute number of CUG codons. The SCU_{CUG} of a gene containing a single Ser residue encoded by a CUG is 1.0, whereas the SCU_{CUG} of a gene with 2 CUGs and 20 Ser residues is 0.1. In order to obtain a global picture of CUG usage in C. *albicans* and to identify functional classes of genes with enrichment or depletion of CUG codons, a global survey of SCU_{CUG} values in different gene ontology (GO) lists was carried out (Tables 1 and 2). Genes encoding plasma membrane proteins differ the most in CUG usage (Table 1). Most interestingly, CUG usage in this category is twofold higher than average CUG usage. Similar CUG usage bias was found in genes encoding nuclear membrane proteins and in genes encoding proteins of the SAGA complex, which is a large multiprotein complex with histone acetyltransferase activity involved in regulation of transcription (e.g., Gcn5p [12]). Conversely, genes encoding proteins of the spindle pole that are involved in the organization of the cytoskeleton and genes of the CCR4-NOT complex, which is involved in transcription regulation, mRNA degradation, and posttranscriptional modifications (32), have a negative CUG usage bias, suggesting that it may be under negative selection (Table 2). CUG codons are also repressed in

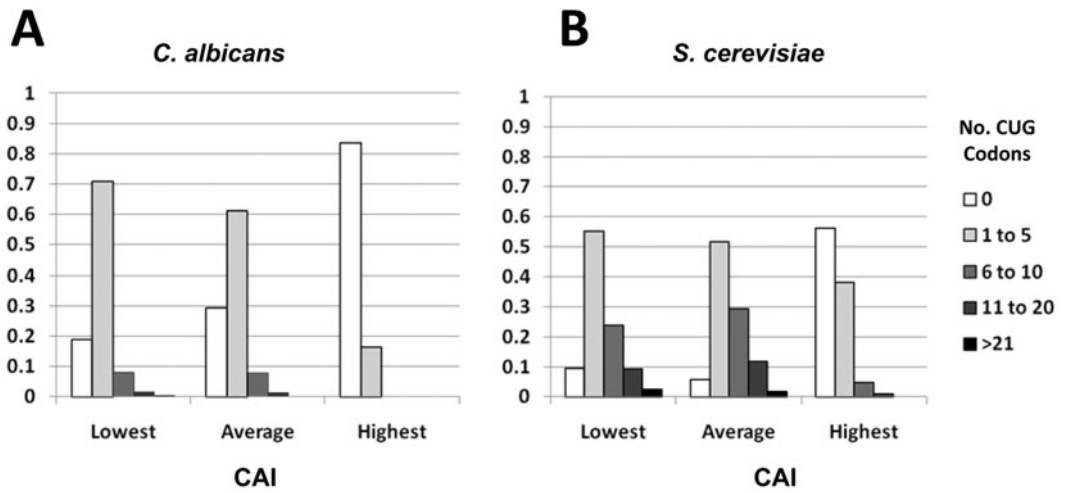


FIGURE 8 CUG usage variation according to gene expression level. Shown is the distribution of CUG codons per gene according to their CAI ranking order in C. *albicans* (A) and *S. cerevisiae* (B). In C. *albicans*, CUG codons are strongly repressed in the 10% of genes with highest CAI values. A similar repression trend is observed in *S. cerevisiae*, but that codon repression is weaker than in C. *albicans*. C. *albicans* genes with lower expression levels accumulate higher numbers of CUG codons. 10.1128/9781555817176.ch4

ribosomal protein genes. However, these proteins are highly expressed and CUG repression may be related to expression level rather than protein function.

In general, CUG usage is repressed in genes related to translational processes and metabolic pathways and is strongly repressed in genes encoding proteins involved in ATP synthesis coupled to proton transport, carbohydrate metabolism, heme biosynthesis, ubiquitin-dependent protein catabolism, and fatty acid catabolism and in genes of NAD^+ biosynthesis, aging processes, processing of 20S pre-rRNA, drug susceptibility and resistance, endocytosis, G_1/S transition of the mitotic cell cycle, DNA replication, and amino acid metabolism. CUG usage shows positive bias in genes of cyclin catabolism, chromatin modification,

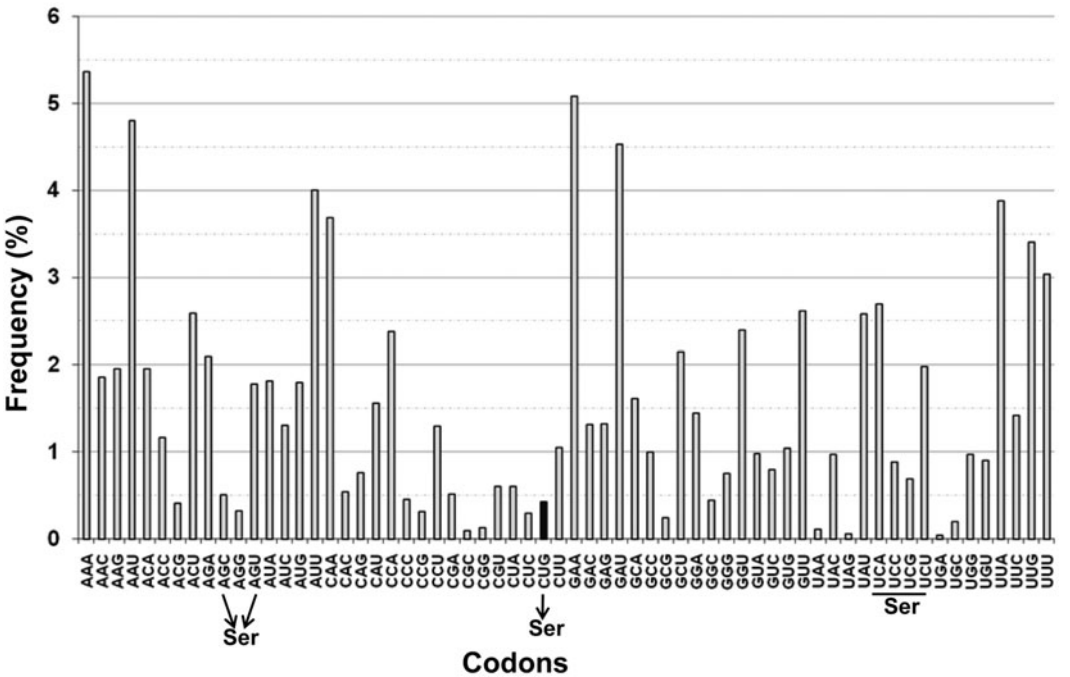


FIGURE 9 Codon usage in C. *albicans*. The CUG is a rare codon in C. *albicans* (0.43%). It is the least used of the seen serine codons. The total codon count was obtained from C. *albicans* genome assembly 19 using ANACONDA (36). 10.1128/9781555817176.ch4f9

TABLE 1 GO terms of genes with highest SCU_{CUG}[a]

GO term	SCU_{CUG}	No. of genes
C:extrinsic membrane protein (ISS)	0.094035	12
P:Golgi to endosome transport (ISS)	0.090082	11
C:spliceosome complex (ISS)	0.078164	18
P:DNA repair (IEA)	0.070072	42
C:centromere (ISS)	0.069168	6
P:regulation of redox homeostasis (ISS)	0.068056	10
C:nuclear membrane (ISS)	0.067259	11
C:AP-N adaptor complex (ISS)	0.064288	14
P:Golgi to vacuole transport (ISS)	0.063687	15
C:DNA replication factor complex	0.062919	10
P:chromatin silencing (ISS)	0.062292	22
C:SAGA complex (ISS)	0.061324	17
C:CCR4-NOT complex (ISS)	0.060933	11
P:mRNA splicing (IEA)	0.059021	54
C:spindle pole (ISS)	0.057906	15
C:chromatin (ISS)	0.057804	29
P:Golgi to plasma membrane transport (ISS)	0.057105	16
P:mitosis (IEA)	0.056839	36
P:cytokinesis (IEA)	0.056295	16
P:protein targeting (ISS)	0.055899	37

[a]The *C. albicans* genes were grouped according to their GO terms, and the average usage of the CUG codon (SCU_{CUG}) of each group was determined. The usage of the CUG codon is increased in genes belonging to the GO terms shown in this table, suggesting that an increased number of combinatorial protein molecules arising from their translation, due to ambiguous CUG codon decoding, could bring a selective advantage to the cell. ISS, inferred from sequence or structural similarity; IEA, inferred from electronic annotation.

TABLE 2 GO terms of genes with lowest SCU_{CUG}[a]

GO term	SCU_{CUG}	No. of genes
C:ribosome (ISS)	0.005718	21
C:cytosolic ribosome (sensu Eukarya) (ISS)	0.006106	94
P:ATP synthesis coupled proton transport (ISS)	0.006250	10
C:respiratory chain complex	0.010942	15
P:carbohydrate metabolism (ISS)	0.015418	10
P:chromatin assembly/disassembly (ISS)	0.016103	13
C:hydrogen-transporting ATPase	0.018612	23
C:extracellular (ISS)	0.018919	6
P:protein biosynthesis (ISS)	0.019061	158
P:ergosterol biosynthesis (ISS)	0.019336	24
C:DNA-directed RNA polymerase	0.020329	35
C:proteasome	0.020918	43
C:RNase complex (ISS)	0.024257	18
P:glycogen metabolism (ISS)	0.024944	12
P:ubiquitin-dependent protein catabolism (ISS)	0.025068	32
C:peroxisome	0.025372	40
P:endocytosis (IEA)	0.025463	18
C:eukaryotic translation initiation factor complex (ISS)	0.026163	10
P:heme biosynthesis (ISS)	0.026417	11
P:35S primary transcript processing (ISS)	0.026831	46

[a]The usage of the CUG codon is repressed in genes belonging to the GO terms shown, indicating a possible negative effect of CUG ambiguity in the gene products.

Golgi-to-endosome transport, cell growth and/or maintenance, budding, mRNA processing, and DNA repair.

CONCLUSIONS

The CTG clade species use a mutant $tRNA_{CAG}^{Ser}$ to decode CUG codons as Ser. The tDNA gene encoding this $tRNA_{CAG}^{Ser}$ appeared before the separation of the *Saccharomyces* and *Candida* genera, and the latter competed for approximately 100×10^6 years with the wild-type $tRNA_{CAG}^{Leu}$ for CUG codons at the ribosome A-site (23, 59). It was lost in the ancestral lineage of *Saccharomyces* spp., where the identity of the CUG codon retained the standard meaning. Conversely, the ancestor of the CTG clade species lost the tDNA gene encoding the cognate $tRNA_{CAG}^{Leu}$ and retained the tDNA gene encoding the mutant $tRNA_{CAG}^{Ser}$, thus changing the identity of the CUG codon from Leu to Ser. This CUG codon identity change imposed strong negative pressure on CUG usage and resulted in massive mutational change of CUG codons to UUG or UUA Leu codons. Indeed, 98% of the CUG codons present in the CTG clade species were erased from their genomes (23). Simultaneously, $tRNA_{CAG}^{Ser}$ captured new CUG codons from the Ser UCN codon family (23), which remained ambiguous over time. The double identity of the CUG codon implies that each protein is represented by a mixture of molecules containing Leu or Ser at CUG positions. This creates a statistical proteome whose biological implications are still poorly understood. Nevertheless, the probability of finding identical cells in nature is extremely small. How *Candida* cells regulate such ambiguous decoding and whether it has an impact on the biology of the CTG clade species are important subjects for future studies.

REFERENCES

1. **Alvarez, F., C. Robello, and M. Vignali.** 1994. Evolution of codon usage and base contents in kinetoplastid protozoans. *Mol. Biol. Evol.* **11:**790–802.
2. **Berman, J., and P. E. Sudbery.** 2002. *Candida albicans*: a molecular revolution built on lessons from budding yeast. *Nat. Rev. Genet.* **3:**918–930.
3. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J. L. Reedy, I. Agrafioti, M. B. Arnaud, S. Bates, A. J. P. Brown, S. Brunke, M. C. Costanzo, D. A. Fitzpatrick, P. W. J. de Groot, D. Harris, L. L. Hoyer, B. Hube, F. M. Klis, C. Kodira, N. Lennard, M. E. Logue, R. Martin, A. M. Neiman, E. Nikolaou, M. A. Quail, J. Quinn, M. C. Santos, F. F. Schmitzberger, G. Sherlock, P. Shah, K. A. T. Silverstein, M. S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M. P. H. Stumpf, P. E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N. A. R. Gow, M. C. Lorenz, B. W. Birren, M. Kellis, and C. A. Cuomo.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459:**657–662.
4. **Calderone, R. A., and W. A. Fonzi.** 2001. Virulence factors of *Candida albicans*. *Trends Microbiol.* **9:**327–335.
5. **Chiapello, H., E. Ollivier, C. Landes-Devauchelle, P. Nitschke, and J. L. Risler.** 1999. Codon usage as a tool to predict the cellular location of eukaryotic ribosomal proteins and aminoacyl-tRNA synthetases. *Nucleic Acids Res.* **27:**2848–2851.
6. **Edelmann, P., and J. Gallant.** 1977. Mistranslation in *E. coli*. *Cell* **10:**131–137.
7. **Ellis, J. T., and D. A. Morrison.** 1995. *Schistosoma mansoni*: patterns of codon usage and bias. *Parasitology* **110**(Pt. 1)**:**53–60.
8. **Enjalbert, B., A. Nantel, and M. Whiteway.** 2003. Stress-induced gene expression in *Candida albicans*: absence of a general stress response. *Mol. Biol. Cell* **14:**1460–1467.
9. **Fitzpatrick, D. A., M. E. Logue, J. E. Stajich, and G. Butler.** 2006. A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. *BMC Evol. Biol.* **6:**99.
10. **Ghaemmaghami, S., W. K. Huh, K. Bower, R. W. Howson, A. Belle, N. Dephoure, E. K. O'Shea, and J. S. Weissman.** 2003. Global analysis of protein expression in yeast. *Nature* **425:**737–741.
11. **Gomes, A. C., I. Miranda, R. M. Silva, G. R. Moura, B. Thomas, A. Akoulitchev, and M. A. Santos.** 2007. A genetic code alteration generates a proteome of high diversity in the human pathogen *Candida albicans*. *Genome Biol.* **8:**R206.
12. **Grant, P. A., D. Schieltz, M. G. Pray-Grant, D. J. Steger, J. C. Reese, J. R. Yates III, and J. L. Workman.** 1998. A subset of TAF_{II}s are integral components of the SAGA complex required for nucleosome acetylation and transcriptional stimulation. *Cell* **94:**45–53.
13. **Ikemura, T.** 1982. Correlation between the abundance of yeast transfer RNAs and the occurrence of the respective codons in protein genes. Differences in synonymous codon choice patterns of yeast and *Escherichia coli* with reference to the abundance of isoaccepting transfer RNAs. *J. Mol. Biol.* **158:**573–597.
14. **Ikemura, T.** 1985. Codon usage and tRNA content in unicellular and multicellular organisms. *Mol. Biol. Evol.* **2:**13–34.
15. **Kano, A., Y. Andachi, T. Ohama, and S. Osawa.** 1991. Novel anticodon composition of transfer RNAs in *Micrococcus luteus*, a bacterium with a high genomic G + C content. Correlation with codon usage. *J. Mol. Biol.* **221:**387–401.
16. **Kawaguchi, Y., H. Honda, J. Taniguchi-Morimura, and S. Iwasaki.** 1989. The codon CUG is read as serine in an asporogenic yeast *Candida cylindracea*. *Nature* **341:**164–166.
17. **Kimata, Y., and M. Yanagida.** 2004. Suppression of a mitotic mutant by tRNA-Ala anticodon mutations that produce a dominant defect in late mitosis. *J. Cell Sci.* **117:**2283–2293.
18. **Knight, R. D., S. J. Freeland, and L. F. Landweber.** 2001. Rewiring the keyboard: evolvability of the genetic code. *Nat. Rev. Genet.* **2:**49–58.
19. **Kurland, C., and J. Gallant.** 1996. Errors of heterologous protein expression. *Curr. Opin. Biotechnol.* **7:**489–493.
20. **Ladner, J. E., A. Jack, J. D. Robertus, R. S. Brown, D. Rhodes, B. F. Clark, and A. Klug.** 1975. Structure of yeast phenylalanine transfer RNA at 2.5 A resolution. *Proc. Natl. Acad. Sci. USA* **72:**4414–4418.
21. **Li, M., and A. Tzagoloff.** 1979. Assembly of the mitochondrial membrane system: sequences of yeast mitochondrial valine and an unusual threonine tRNA gene. *Cell* **18:**47–53.
22. **Lovett, P. S., N. P. Ambulos, Jr., W. Mulbry, N. Noguchi, and E. J. Rogers.** 1991. UGA can be decoded as tryptophan at low efficiency in *Bacillus subtilis*. *J. Bacteriol.* **173:**1810–1812.
23. **Massey, S. E., G. Moura, P. Beltrao, R. Almeida, J. R. Garey, M. F. Tuite, and M. A. Santos.** 2003. Comparative evolutionary genomics unveils the molecular mechanism of reassignment of the CTG codon in *Candida* spp. *Genome Res.* **13:**544–557.
24. **Matsugi, J., K. Murao, and H. Ishikura.** 1998. Effect of *B. subtilis* $tRNA^{Trp}$ on readthrough rate at an opal UGA codon. *J. Biochem.* **123:**853–858.
25. **Miranda, I., R. Rocha, M. C. Santos, D. D. Mateus, G. R. Moura, L. Carreto, and M. A. Santos.** 2007. A genetic

code alteration is a phenotype diversity generator in the human pathogen *Candida albicans*. *PLoS One* **2:**e996.
26. **Moura, G. R., T. Lima-Costa, L. Carreto, A. C. Gomes, and M. A. Santos.** 2007. Molecular evolution of the *Candida* genetic code. *In* C. d'Enfert and B. Hube (ed.), Candida—*Comparative and Functional Genomics*. Caister Academic Press, Norfolk, United Kingdom.
27. **Oba, T., Y. Andachi, A. Muto, and S. Osawa.** 1991. CGG: an unassigned or nonsense codon in *Mycoplasma capricolum*. *Proc. Natl. Acad. Sci. USA* **88:**921–925.
28. **Ogle, J. M., and V. Ramakrishnan.** 2005. Structural insights into translational fidelity. *Annu. Rev. Biochem.* **74:**129–177.
29. **Ohama, T., A. Muto, and S. Osawa.** 1990. Role of GC-biased mutation pressure on synonymous codon choice in *Micrococcus luteus*, a bacterium with a high genomic GC-content. *Nucleic Acids Res.* **18:**1565–1569.
30. **Osawa, S., and T. H. Jukes.** 1989. Codon reassignment (codon capture) in evolution. *J. Mol. Evol.* **28:**271–278.
31. **Osawa, S., T. H. Jukes, K. Watanabe, and A. Muto.** 1992. Recent evidence for evolution of the genetic code. *Microbiol. Rev.* **56:**229–264.
32. **Panasenko, O., E. Landrieux, M. Feuermann, A. Finka, N. Paquet, and M. A. Collart.** 2006. The yeast Ccr4-Not complex controls ubiquitination of the nascent-associated polypeptide (NAC-EGD) complex. *J. Biol. Chem.* **281:**31389–31398.
33. **Parker, J.** 1989. Errors and alternatives in reading the universal genetic code. *Microbiol. Rev.* **53:**273–298.
34. **Perreau, V. M., G. Keith, W. M. Holmes, A. Przykorska, M. A. Santos, and M. F. Tuite.** 1999. The *Candida albicans* CUG-decoding ser-tRNA has an atypical anticodon stem-loop structure. *J. Mol. Biol.* **293:**1039–1053.
35. **Perry, J., X. Dai, and Y. Zhao.** 2005. A mutation in the anticodon of a single $tRNA^{ala}$ is sufficient to confer auxin resistance in *Arabidopsis*. *Plant Physiol.* **139:**1284–1290.
36. **Pinheiro, M., V. Afreixo, G. Moura, A. Freitas, M. A. Santos, and J. L. Oliveira.** 2006. Statistical, computational and visualization methodologies to unveil gene primary structure features. *Methods Inf. Med.* **45:**163–168.
37. **Pouwels, P. H., and J. A. Leunissen.** 1994. Divergence in codon usage of *Lactobacillus* species. *Nucleic Acids Res.* **22:**929–936.
38. **Ruan, B., S. Palioura, J. Sabina, L. Marvin-Guy, S. Kochhar, R. A. Larossa, and D. Soll.** 2008. Quality control despite mistranslation caused by an ambiguous genetic code. *Proc. Natl. Acad. Sci. USA* **105:**16502–16507.
39. **Santos, M. A., C. Cheesman, V. Costa, P. Moradas-Ferreira, and M. Tuite.** 1999. Selective advantages created by codon ambiguity allowed for the evolution of an alternative genetic code in *Candida* spp. *Mol. Microbiol.* **31:**937–947.
40. **Santos, M. A., G. Keith, and M. F. Tuite.** 1993. Non-standard translational events in *Candida albicans* mediated by an unusual seryl-tRNA with a 5′-CAG-3′ (leucine) anticodon. *EMBO J.* **12:**607–616.
41. **Santos, M. A., G. Moura, S. E. Massey, and M. F. Tuite.** 2004. Driving change: the evolution of alternative genetic codes. *Trends Genet.* **20:**95–102.
42. **Santos, M. A., V. M. Perreau, and M. F. Tuite.** 1996. Transfer RNA structural change is a key element in the reassignment of the CUG codon in *Candida albicans*. *EMBO J.* **15:**5060–5068.
43. **Santos, M. A., and M. F. Tuite.** 1995. The CUG codon is decoded in vivo as serine and not leucine in *Candida albicans*. *Nucleic Acids Res.* **23:**1481–1486.
44. **Santos, M. A., T. Ueda, K. Watanabe, and M. F. Tuite.** 1997. The non-standard genetic code of *Candida* spp.: an evolving genetic code or a novel mechanism for adaptation? *Mol. Microbiol.* **26:**423–431.
45. **Schultz, D. W., and M. Yarus.** 1994. Transfer RNA mutation and the malleability of the genetic code. *J. Mol. Biol.* **235:**1377–1380.
46. **Schultz, D. W., and M. Yarus.** 1996. On malleability in the genetic code. *J. Mol. Evol.* **42:**597–601.
47. **Sengupta, S., and P. G. Higgs.** 2005. A unified model of codon reassignment in alternative genetic codes. *Genetics* **170:**831–840.
48. **Sharp, P. M., and W. H. Li.** 1986. An evolutionary perspective on synonymous codon usage in unicellular organisms. *J. Mol. Evol.* **24:**28–38.
49. **Silva, R. M., J. A. Paredes, G. R. Moura, B. Manadas, T. Lima-Costa, R. Rocha, I. Miranda, A. C. Gomes, M. J. Koerkamp, M. Perrot, F. C. Holstege, H. Boucherie, and M. A. Santos.** 2007. Critical roles for a genetic code alteration in the evolution of the genus *Candida*. *EMBO J.* **26:**4555–4565.
50. **Soma, A., R. Kumagai, K. Nishikawa, and H. Himeno.** 1996. The anticodon loop is a major identity determinant of *Saccharomyces cerevisiae* $tRNA^{Leu}$. *J. Mol. Biol.* **263:**707–714.
51. **Sprinzl, M., C. Horn, M. Brown, A. Ioudovitch, and S. Steinberg.** 1998. Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.* **26:**148–153.
52. **Stansfield, I., K. M. Jones, P. Herbert, A. Lewendon, W. V. Shaw, and M. F. Tuite.** 1998. Missense translation errors in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **282:**13–24.
53. **Sugita, T., and T. Nakase.** 1999. Non-universal usage of the leucine CUG codon and the molecular phylogeny of the genus *Candida*. *Syst. Appl. Microbiol.* **22:**79–86.
54. **Suzuki, T., T. Ueda, and K. Watanabe.** 1997. The 'polysemous' codon—a codon with multiple amino acid assignment caused by dual specificity of tRNA identity. *EMBO J.* **16:**1122–1134.
55. **Tuite, M. F., P. A. Bower, and C. S. McLaughlin.** 1986. A novel suppressor tRNA from the dimorphic fungus *Candida albicans*. *Biochim. Biophys. Acta* **866:**26–31.
56. **Turanov, A. A., A. V. Lobanov, D. E. Fomenko, H. G. Morrison, M. L. Sogin, L. A. Klobutcher, D. L. Hatfield, and V. N. Gladyshev.** 2009. Genetic code supports targeted insertion of two amino acids by one codon. *Science* **323:**259–261.
57. **Woo, N. H., B. A. Roe, and A. Rich.** 1980. Three-dimensional structure of *Escherichia coli* initiator tRNAf-Met. *Nature* **286:**346–351.
58. **Yokobori, S., T. Suzuki, and K. Watanabe.** 2001. Genetic code variations in mitochondria: tRNA as a major determinant of genetic code plasticity. *J. Mol. Evol.* **53:**314–326.
59. **Yokogawa, T., T. Suzuki, T. Ueda, M. Mori, T. Ohama, Y. Kuchino, S. Yoshinari, I. Motoki, K. Nishikawa, S. Osawa, and K. Watanabe.** 1992. Serine tRNA complementary to the nonuniversal serine codon CUG in *Candida cylindracea*: evolutionary implications. *Proc. Natl. Acad. Sci. USA* **89:**7408–7411.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch5

5

Genome Instability and DNA Repair

GERMÁN LARRIBA AND RICHARD CALDERONE

INTRODUCTION

Candida albicans is a diploid yeast that causes opportunistic infections in humans. There is growing evidence indicating that during the infection process, the success of the organism relies to a large extent on its ability to adapt to the several niches in the human host through genetic changes, mainly point mutation, loss of heterozygosity (LOH), and aneuploidies (51, 56, 72). The genetic plasticity of *C. albicans* is likely derived, at least in part, from its obligate diploidy (21, 65, 163) and is manifested in the karyotype variability found among the clinical isolates. As noted previously, these genetic changes may also contribute to the maintenance of a commensal and opportunistic life history of *C. albicans* (51). Since mutation, LOH, and aneuploidy rates are under the control of the DNA replication/repair/recombination machinery, the analysis of mutation/recombination processes and mechanisms may help to understand the biology and pathogenesis of *C. albicans*. Recombination events not only make possible genomic rearrangements useful to this fungus but also, at the same time, suppress mutagenic rearrangements that cause unnecessary genetic instability and, as a consequence, a decrease in its invasive potential. Exacerbation of the natural genetic instability potentially could be used to decrease the ability of *C. albicans* to compete in the commensal environment or to survive in patients. Also, because of its obligate diploidy, *C. albicans* is a better model than *Saccharomyces cerevisiae* to correlate this kind of study with human cells, in which aneuploidy has been proposed to initiate tumor formation. In this chapter, we also describe DNA repair systems that have not been described for *Candida* species even though orthologues are found at least in the *C. albicans* genome databases. A section of this chapter also describes the genetic plasticity as it relates to drug resistance. For additional reading, see chapter 23.

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GENETIC INSTABILITY

Genome Instability: Heterozygosity, LOH, and Point Mutation

C. albicans is an obligate diploid whose genome has a high level of polymorphisms, including more than 55,000 single nucleotide polymorphisms (SNPs) in the reference strain SC5314. This translates to 1 SNP per about 300 bp, one of the highest densities so far found in a sequenced genome, including those of highly related *Candida* species (25). Distribution of these SNPs is not homogeneous, since cold and hot polymorphic regions are found in the genome. Also, some of the SNPs are in intergenic regions, whereas others are inside the open reading frames (ORFs). Noticeably, about 2,792 ORFs (from more than 6,000) carry nonsynonymous SNPs, which cause changes in the amino acid sequence of the encoded protein (81, 87, 105, 160, 187). Therefore, there are two versions of nearly half of all proteins encoded by the genome of *C. albicans*, which differ in one to a few amino acids.

Variation of the SNPs between isolates is a hallmark of the genetic variability of the species and the basis of strain typing methods, including SNP array analysis (52, 54) and multilocus sequence typing (MLST) (22–24, 185). MLST analyzes the sequences of 7 to 10 well-conserved housekeeping genes. Each allele is given a number, and therefore, each strain is defined with a bar code or diploid sequence type (DST). Computational analysis of the MLST data derived from 7 genes from each of 1,410 DSTs, which carried a total of 170 SNPs, has allowed the elaboration of dendrograms that define phylogenetic relationships. The corresponding strains are grouped into five large clades, although each gene yields a different phylogenetic tree (129, 130). Most DST are represented by one or a few isolates, but some of them have been found in more than 10 isolates, and DST69 from clade 1 was found in more than 100 isolates (128, 129). However, it is likely that the isolates belonging to one DST are not fully identical but differ in SNPs from other genes. If so, each isolate may be unique.

In an organism with high levels of heterozygosity, genes or chromosomal segments may become homozygotic following homologous recombination (HR) or other genetic processes. These processes, known as LOH, occur spontaneously

at a frequency of 10^{-4} to 10^{-6} (51, 94). The LOH rate is a measurement of the recombinational activity of the cell and therefore of its genetic instability. The mechanisms of LOH that may occur in *C. albicans* have been described before (87, 163; see also below, "The Genetic Basis of Instability: Mechanisms of DNA Repair in *C. albicans*"). The application of different typing techniques, including DNA fingerprinting and MLST, to clinical isolates has indicated that following colonization of different individuals by the same strain, LOH events, likely derived from gene conversion (GC), lead to microevolution (53, 98). Similarly, isolates from different sites or from the same site but at different times from each patient are highly related, yet they still frequently show small changes (microvariations) due to LOH events (22, 130). Thus, LOH largely contributes to the expansion of DSTs and, therefore, to the variability/diversity of *C. albicans* (128, 129).

LOH has a substantial effect on the speed of adaptation of diploid asexual populations, and the appearance of a beneficial dominant or codominant mutation in a heterozygote is rapidly followed by the production of a mutant homozygote (108). It is expected that when the single protein encoded by the homozygous version of the affected gene(s) confers a large fitness advantage under a specific condition(s) (adaptive mutation), that clone will outcompete other variants and become dominant in the corresponding environment. Therefore, by converting heterozygotes to homozygotes, LOH contributes to the spread of beneficial alleles in *C. albicans*. These dynamic changes are not restricted to *C. albicans*. Mitotic recombination during the diploid phase of the parasexual cycle accelerates adaptation of *Aspergillus nidulans* mediated, at least in part, by LOH events (161). In *C. albicans*, LOH rates seem to be influenced by environmental factors. For instance, for at least the *GAL1* locus, recombination rates significantly increased during infection (28-fold) compared to in vitro propagation (51). This observation fits the general concept that a heterogeneous environment broadens the conditions that favor higher recombination rates (93).

Genetic population studies using the data derived from the several typing methods, including restriction fragment length polymorphism with the Ca3 probe and MLST, indicate that in spite of the surprising genetic variability of the species, *C. albicans* reproduces mainly by clonal propagation with low doses of recombination (63, 129, 146). This is inferred from the excess of heterozygosity found among the isolates analyzed. Deviations of the Hardy-Weinberg expectations associated with an excess of heterozygosity or homozygosity are incompatible with frequent sexual exchange (63, 128). There is evidence for a parasexual cycle that involves mating of two diploid cells (*MTL***a** and *MTL*α) to form a tetraploid. Under stress conditions, the tetraploid undergoes a process of concerted chromosome loss that leads to a diploid or quasidiploid state, but meiosis has not been described (19). Furthermore, same-sex mating between *MTL*-homozygous strains of *C. albicans* may also occur, and the resulting tetraploid cell also returns to the diploid state through chromosome loss (4). This parasexual cycle may generate new combinations of the highly polymorphic chromosomes present in the mating partners including new pairs of homologues and shuffling of the nonhomologues. In addition, significant levels of LOH promoted by HR occur during the parasexual cycle induced in the laboratory (52). However, the real extent of the contribution of this process to the creation of diversity is uncertain, because the frequency at which conjugation occurs in nature is unknown. Regardless of its occurrence during clonal (mitotic) or parasexual reproduction, interhomologue (as opposed to intersister chromatid) HR plays an important role in the generation of diversity, since it may produce recombinant alleles (intragenic recombination) or a new combination of alleles of genes located in the same chromosome (intergenic recombination). In addition to creating variability, HR is an important mechanism to repair DNA damage in *C. albicans* (32, 59, 91), which is not surprising since, because of its obligate diploidy, *C. albicans* always has a homologous partner to engage in recombination (see below).

HR tends to decrease the heterozygosity of the species. Still, isolates conserve a high level of heterozygosity. According to recent published data, the sequenced strains SC5314 and WO-1 have 1 SNP per 330 and 390 bp, respectively (25; see also chapter 3). However, SNPs are not conserved but differ among isolates. For instance, strains SC5314 and WO-1 share only 2 to 3% of the SNPs, in spite of the fact that their genomes are largely colinear, with the exception of 12 inversions between them (25). Similarly, several laboratory strains differ in SNPs present in the *HIS4* ORF (61). These observations suggest that *C. albicans* has mechanisms to regain heterozygosity, likely through point mutations which counteract LOH caused by mitotic recombination. In *S. cerevisiae* haploid cells, the rate of spontaneous mutation in the nuclear genome is rather low under laboratory conditions (100). However, no studies on the rate of accumulation of neutral mutations by *C. albicans* under laboratory conditions or during the infectious process have been reported. Recent results with *S. cerevisiae* indicate that an increase in frequency of GC is accompanied by an increase in the mutation rate. Mutagenesis was especially promoted by microhomology-mediated template switches and was caused by errors made by polymerases δ and ε (70). Therefore, in *C. albicans* the spontaneous-mutation rate might benefit from LOH events.

There is a large body of theory and experimental evidence indicating that a diploid organism can undergo more mutations than a haploid because a diploid has twice the number of mutation targets as a haploid. In addition, deleterious mutations are better supported in a diploid than in a haploid because they can be masked (if they are recessive) by the heterozygous state (132). Similarly, diploids support better exposure to mutagens (102). The heterozygous state also has the advantage of carrying two allelic versions that can diverge in function or regulation. Several studies have provided evidence for this in *C. albicans* (45, 46, 53, 61, 175; see reference 87 for a review). Importantly, recent studies suggest that beneficial mutations in asexual populations are more frequent than previously thought, and several of them occur in the same lineage and are fixed simultaneously (26, 41). On the other hand, as noted above, LOH is an important factor in the spread of beneficial alleles, and this process will benefit from increasing mitotic recombination rates. In this regard, experimental evidence from *S. cerevisiae* indicates that diploids adapt better to environments that require dominant adaptive mutations (11). We suggest that both point mutation and LOH are important sources of genetic instability in *C. albicans* and that they may contribute extensively to the variability and evolution of the fungus, especially during the infection process, when the host environment provides variable challenges.

Genome Instability: Aneuploidies

An additional marker of the genetic instability in *C. albicans* is represented by aneuploidies. Although the standard

karyotype of *C. albicans* has eight pairs of homologues (172), near half of the clinical isolates frequently show karyotypic variations (78, 88, 104, 123, 155, 172). Some of the variations are due to reciprocal translocations at the level of the major repeat sequence (MRS), as shown for the WO-1 strain (31). A diagram illustrating how this process may occur is shown in Fig. 1A (30, 104, 105). Karyotype variation is due to chromosomal length polymorphisms derived from variations in the number of a 2-kb repeat (RPS) in the MRS on one homologue compared to the other (78, 95; for reviews, see references 30 and 105). Early in vitro studies also demonstrated the presence of chromosomal alterations in spontaneous morphological (17, 154, 156, 183) and nutritional (77, 152, 153, 183) mutants of *C. albicans*, and gross chromosomal rearrangements (GCR) of a different nature have been found more recently and shown to be associated with fluconazole resistance (Flu^R) (164, 165, 167). Karyotype variability has been also reported for clinical isolates of *Candida dubliniensis* (103).

Aneuploidies are common in laboratory strains of *C. albicans* (3, 29, 166) but are especially abundant when those strains have been subjected to genetic manipulations, including several laboratory strains successively derived from CAI-4, or treated with mutagenic agents such as UV light (7, 12, 21, 61; for reviews, see references 150, 151, and 163, the last for a summary of the genetic changes in several strains). A well-known example is provided by the 23-kb terminal deletion in the right arm of chromosome 5 of strain BWP17, a CAI-4 derivative. This truncation occurred during disruption of *HIS1* in one of its progenitors, RM1000#6 (5, 54, 164). There is direct evidence that aneuploidies may be even caused by heat shock and transformation methods using either lithium acetate or electroporation and that different in vitro stresses may cause different aneuploidies in *C. albicans* (21). In another study, fluconazole selected for strains that were either monosomic for chromosome 4 or trisomic for chromosome 3 (138).

Karyotype alterations also occur during infection and are more common in isolates from deep sites of infection (42, 51, 53, 164), suggesting that strains carrying aneuploidies support a stronger selective pressure. A comparison of the frequency of genetic changes in vitro and in vivo (i.e., during passage through a living host) is difficult because in vivo, the growth rate is 5- to 10-fold lower. Recent experiments indicate that short-range LOH (from individual SNPs to <300-bp segments) occurs at similar rates (events/cell/generation) during propagation of *C. albicans* populations in vitro and in vivo. Yet, LOH involving entire chromosomes and other aneuploidies were observed only among the populations propagated in vivo. The latter alterations frequently correlate with an altered colonial morphology and were presumably induced by the stress conditions prevalent during infection (51).

The high tolerance of *C. albicans* to aneuploidies is probably due in part to its diploid nature. Studies with *S. cerevisiae* indicate that following irradiation, about two-thirds of the colonies of diploid cells yielded new extrachromosomal bands, whereas only a few percent of haploid cells showed karyotype alterations, suggesting that diploid cells can better support GCR that are deleterious in haploids (13). Also, haploid cells selected under glucose-limited conditions exhibited aneuploidies of primarily short segments of the genome (44, 65, 83) that caused growth defects under nonselective conditions, likely due to the subsequent imbalance of gene products (186). Compared to haploids, *S. cerevisiae* diploids experienced higher rates of aneuploidy that, in turn, allowed adaptation to nutrient deprivation (65). However, a further increase in ploidy, from diploid to tetraploid cells, creates new requirements for maintenance of genomic stability and fitness, including a stronger requirement for HR, sister chromatid cohesion, and function of the spindle body (177). In agreement with this, *C. albicans* tetraploids were less virulent than diploids in a model of disseminated candidiasis in mice (74).

It should be noted that factors other than diploidy should also influence tolerance to aneuploidy. For instance, in the presence of fluconazole, *C. albicans* had a higher frequency of chromosome rearrangements than did an *S. cerevisiae* diploid strain (11, 167). Also, the asexual haploid yeast *Candida glabrata* undergoes a variety of GCR, including reciprocal and nonreciprocal translocations, interchromosomal duplications, chromosome fusions, and segmental duplications followed by addition of telomeres, although whole chromosome aneuploidies were not reported. Importantly, some of

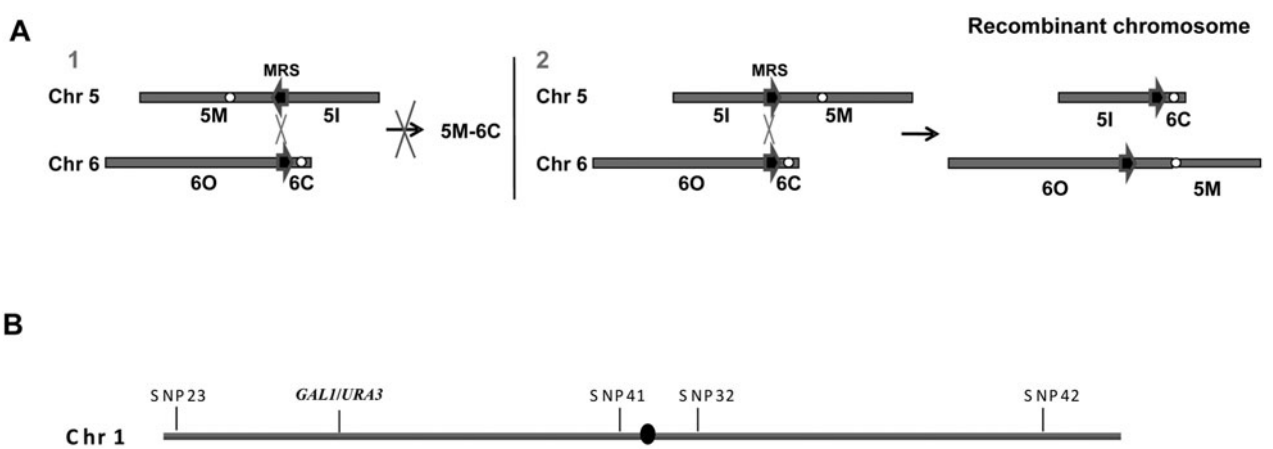


FIGURE 1 (A) Mechanism of reciprocal translocations mediated by MRS. MRS, when acting as direct repeats, but not as inverted repeats, may cause chimerical chromosomes. The chimerical chromosomes shown have been found in strain WO-1. (B) Scheme of Chr1 and the genetic system used to determine recombination rates in *C. albicans* (for details, see text). 10.1128/9781555817176.ch5f1

these rearrangements were connected with antifungal drug resistance and stress during infection (141, 168).

How can genomic rearrangements and aneuploidies adapt *C. albicans* to the stress encountered in vitro and in vivo? The response of *C. albicans* to sorbose has been analyzed in depth by Rustchenko and coworkers. Mutants able to grow in sorbose as the only carbon source (Sou^+) are monosomic for chromosome 5, suggesting that growth on sorbose selects for loss of one copy of chromosome 5. Conversely, Sou^+ mutants revert to Sou^- strains when maintained in rich medium; these revertants become disomic for chromosome 5 (79). It has been proposed that monosomy of chromosome 5 results in the activation of the structural gene *SOU1*, responsible for the utilization of sorbose, which is located in chromosome 4. This could occur through downregulation of *SOU1* by negative regulators present in the right arm of chromosome 5 (64, 82). In agreement with this, strains carrying a terminal deletion of the right arm of chromosome 5 can also grow in sorbose (7). These findings constitute an example of a novel general means of gene regulation based on segmental or whole chromosome copy number. More recently, it has been reported that strains adapted to grow in fluconazole are aneuploid, and those most resistant carry an extra isochromosome formed by two left arms of chromosome 5 [i(5L)] (164, 165). Formation of i(5L) results in duplication of *ERG11* (150 kb from the telomere) and *TAC1* (48 kb from the centromere), two genes involved in fluconazole resistance (see below).

THE GENETIC BASIS OF INSTABILITY: MECHANISMS OF DNA REPAIR IN *C. ALBICANS*

Genetic instability could be caused by an increase in the rate of mutations in the form of single base substitutions, microinsertions, and microdeletions. These alterations are known to arise from errors during normal DNA replication by polymerases δ and ε (80) and are usually corrected before being fixed by methyl mismatch repair (MMR). Pyrimidine dimers caused by UV light as well as spontaneous or induced abasic sites are also a source of mutations during the next round of replication, since they are bypassed by error-prone polymerases that introduce mutations into the newly synthesized band (translesion synthesis [TLS]). To avoid these mutations, pyrimidine dimers are removed by either photolyase or nucleotide excision repair (NER), whereas abasic sites are mostly corrected by base excision repair (BER). Therefore, any impairment in these surveillance pathways results in genetic instability mainly in the form of mutations. The presence of minisatellites is a source of genetic instability, since both HR (mainly unequal sister chromatid exchange) and replication slippage processes may cause expansions and contractions of this repetitive DNA (for reviews, see references 1, 2, 68, 136, and 145). Nonallelic HR between repeated sequences may result in a number of GCR. Impairment in the regulation of HR or nonhomologous end joining (NHEJ) may lead to excess LOH or GCR. End joining between two fragments of DNA may result in translocations, and defects in HR may result in a higher rate of mutation, chromosome loss, and chromosome truncation (191). Defects in NHEJ also cause alterations in the length of telomeres. A summary of the pathways responsible for the maintenance of genetic stability, including the genes and enzymes involved in the model organisms *Escherichia coli* and *S. cerevisiae*, is shown in Table 1. Genes from most of these pathways have been identified in *C. albicans* and some of them further characterized. Kirpatrick's group from the University of Minnesota has characterized genes involved in MMR, BER, HR, and NHEJ, whereas our group has studied genes involved in HR and NHEJ.

Methyl Mismatch Repair

In most organisms, many errors that escape proofreading can be detected by the DNA MMR system. This system was first discovered in bacteria, in which it has been investigated in depth. Briefly, a dimer of the protein MutS scans the genome for mismatches, which are recognized because of the distortion they cause in the DNA molecule. Following binding to mismatches, MutS recruits MutL, and the MutS-MutL complex activates the MutH endonuclease, which nicks the newly synthesized band at the 3′ or 5′ adjacent hemimethylated *dam* site (GA*TC). The tetranucleotide GATC occurs every 256 bp on average, and therefore, one hemimethylated *dam* site transiently exists not far away behind the replication fork. This targets the newly synthesized unmethylated band to be resected and removed from the *dam* site to the mismatch by the combined action of an exonuclease and a helicase (for reviews, see references 68, 117, and 174). A polymerase refills the gap. MMR is also involved in the stability of bacterial microsatellites, whose length is altered by DNA polymerase slippage events during replication of these repetitive tracks. Therefore, microsatellites are intrinsically unstable. In the absence of MMR, gain or loss of one or more repeats occurs.

At least six MutS (Msh1p to Msh6p) and four MutL (Mlh1 to Mlh3 and Pms1) homologues have been characterized in *S. cerevisiae* (68, 85). Msh1p is involved in maintenance of genetic stability of mitochondrial DNA; Msh2, Msh3, and Msh4 participate in the stabilization of the nuclear genome; and Msh5 and Msh6 act in meiotic recombination. Mutations in several of these genes caused a significant increase in the rate of spontaneous mutation to canavanine resistance in haploids through base substitutions, frameshift mutations, and microsatellite instability. The MutL homologues Pms1 and Mlh1, and the MutS homologues Msh2 and Msh3, are involved in maintaining the stability of microsatellites whose repeat unit is 1 to 13 bp in length, whereas Msh6p stabilizes 1- to 2-bp repeats. Stability of microsatellites with longer repeats likely uses other pathways. In addition, yeast MMR proteins have an antirecombinogenic activity that is manifested when the identity between the DNA partners engaging in recombination decreases. In fact, some MMR proteins suppressed HR between diverging sequences in *S. cerevisiae* (85).

The orthologues of *PMS1* (a MutL homologue) and *MSH2* (a MutS homologue) have been characterized in *C. albicans*. Null mutants in each of these genes showed a LOH frequency similar to that of wild-type cells (91). The system used to determine LOH rates analyzed two counterselectable genetic markers, *GAL1* and *URA3*, located on opposite alleles at the same locus on chromosome 1 (*GAL1*/*gal1*::*URA3*) (55, 91). This experimental approach takes advantage of the ability of Gal^-, but not Gal^+, strains to grow in the presence of 2-deoxygalactose (2-DG), which allows its selection on 2-DG plates. Alternatively, *GAL1*/*GAL1* derivatives (Uri^-) can be detected as 5-fluoroorotic acid-resistant (FOA^R) clones. An analysis of selected SNP markers located at both sites of the *GAL1* marker allows inference to the mechanisms involved in LOH (Fig. 1B).

TABLE 1 Pathways responsible for maintenance of genetic stability[a]

Repair systems	Damage	Genes/enzymes		
		E. coli	*S. cerevisiae*	*C. albicans*
MMR	Replication errors that escape proofreading	MutS, MutL, MutH	*MSH1-MSH6, MLH1-MLH3, PMS1*	***PMS1*, *MSH2***
Direct reversal of damage				
Photoreactivation	Pyrimidine dimers	DNA photolyase	DNA photolyase	?
Methyl group removal	(Methyl-G) MMS	Alkyltransferase I Alkyltransferase II	*MGT1*	*MGT1* homolog
Base excision repair (BER)	Damaged base	DNA glycosylases	DNA glycosylases *UNG1, OGC1, NTG1, NTG2, MAG1*	***OGG1, NTG1***
		AP endonucleases	AP endonucleases *ANP1, ANP2*	***ANP1***
		AP lyases	AP lyases *NTG1, NTG2, OGG1*	***OGG1*, *NTG1***
Nucleotide excision repair (NER)	Thymine dimers Bulky adduct on base	*uvrA, uvrB, uvrC, uvrD*	*RAD3* epistasis group *RAD1, RAD2, RAD10. . .*	***RAD2*, *RAD10***
Double-strand break repair (DSBR) and other HR pathways	DSBs	*recA* and *recBCD*	*RAD52* epistasis group *RAD51, RAD52, RAD54, RAD55, RAD57, RAD59*	***RAD51*, *RAD52*, *RAD59***
			MRX complex *RAD50, MRX11, XRS2*	***RAD50*, *MRX11***
			Helicases, exonucleases *SAE2, EXO1, SGS1, DNA2*	***SGS1*** **(91a)**
Nonhomologous end joining (NHEJ)	DSBs	*ku, ligD*	*KU70, KU80, LIG4, LIF1, LIF2*	***KU70*, *KU80*, *LIG4***
Translesion synthesis (TLS)	Pyrimidine dimers/AP site	Y-family polymerase (*umuC*)	*REV1, REV3, REV7, RAD30, POL32*	*REV1, REV3, REV, RAD30, POL32*, homologs
			RAD6	***RAD6***

[a]*C. albicans* genes that have been characterized to some extent are in boldface and underlined.

In wild-type cells, LOH was always caused by a reciprocal crossover (CO) or break-induced replication (BIR). However, a significant percentage of the FOA^R derivatives from the C*amsh2* deletion mutant (12.5%) conserved the *URA3* gene and remained heterozygous for the selected SNP markers, suggesting mutational inactivation of *URA3*. Similarly, significant percentages of FOA^R and $2\text{-}DG^R$ strains (12.5%) that conserved *URA3* and *GAL1*, respectively, were found among the *pms1* derivatives. Sequencing of both genes confirmed the presence of mutations. Most of these mutations consisted of a single base insertion or deletions within repetitive DNA tracts, but single base substitutions were also detected. Therefore, *C. albicans* mutants with deletions in MMR genes accumulated point mutations above wild-type levels (91). Interestingly, when exposed to fluconazole, both *msh2* and *pms1* null strains exhibited MICs identical to those of the parental strain but subsequently generated clones that were resistant to higher doses of the drug. Upon retesting, one-third of them had lost the fluconazole resistance and the level of resistance varied among the remaining clones (91). It is likely that this dynamic change is derived from the mutator phenotype of these deletion mutants, which, as described above, can cause insertion or deletion of both single bases within repetitive tracks and repeat units within microsatellites.

From the function of MMR genes, it is expected that organisms carrying relaxed alleles of those genes show an enhanced mutation rate (mutator phenotype). In humans, some polymorphisms cause only weak MMR defects. However, several weak alleles able to interact with other weak (or loss-of-function) alleles in a number of MMR genes have been also identified and shown to cause strong polygenic MMR defects and cancer (110). Whether the occurrence of weak MMR gene alleles is common in *C. albicans* remains to be investigated. However, given the high number of interactions of MMR proteins with each other or other proteins, they are ideal agents to maintain background mutation rates at a level compatible, although maybe in the limit, with the preservation of the genome. As mentioned above, deleterious mutations could be masked in a diploid.

Reversal of DNA Damage

Spontaneous mutations or damage caused by exogenous agents is repaired by other pathways, including reversal of base damage caused by UV light or methylation, BER, NER, and double-strand break repair (DSBR) pathways.

Reversal of Pyridimine Dimers

Pyrimidine dimers caused by UV radiation can be repaired by either photolyases or NER pathways. Photolyases that repair cyclobutane rings are members of a widely extended family of blue-light receptor proteins which are activated by light. The *E. coli* photolyase is the best-characterized enzyme. A homologue of the *E. coli* photolyase has been found in *S. cerevisiae*. The two proteins have very similar amino acid sequences, and transcription of *S. cerevisiae* photolyase is also activated when cells are exposed to UV light (157). Interestingly, searches for homologues in *C. albicans* have been unsuccessful, suggesting that this enzyme has been lost in the *C. albicans* lineage (32, 91).

Reversal of Alkylation

Two proteins mediate adaptation of *E. coli* to grow in the presence of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG). Low doses of MNNG induce resistance of *E. coli* cells to the mutagenic and lethal effects of higher concentrations of the drug (adaptive response). Resistance was due to induction of an O^{6}-alkylguanine-DNA alkyltransferase (also known as Ada, after its role in adaptation), whose methyltransferase activity removes methyl groups from O^{6}-methylguanine. A second activity, known as O^{6}-alkylguanine-DNA alkyltransferase II, provides an immediate response against alkylating damage. This is encoded by the *ogt*$^{+}$ gene, which is distinct from *ada*$^{+}$, although the encoded proteins have extensive regions of homology in their amino acid sequences (reviewed in reference 116).

There is no evidence for an adaptive response to alkylating agents in yeast. In *S. cerevisiae*, a single gene, *MGT1*, is involved in removing methyl groups from O^{6}-methylguanine and, to a lesser extent, from O^{4}-methylthymine, and *MTG1* functionally complements an *ada ogt* double mutant from *E. coli* (190). A homologue of *MGT1* has been identified but not yet characterized in the genome of *C. albicans*.

BER

BER removes bases damaged either spontaneously or by the action of exogenous agents such as alkylating compounds and reactive oxygen species. Modified bases can arise as a consequence of the spontaneous or induced deamination of cytosine or methylcytosine (which leaves uracil or thymine opposite of guanine), oxidized bases (8-oxoG), adenine opposite to 8-oxoG, methyl adenine, ring saturated pyrimidines, etc. In the BER pathway, modified bases are removed by the action of a glycosylase, an enzyme that cleaves the *N*-glycosyl bonds between the base and the deoxyribose moiety that forms the backbone of the DNA molecule. The sites free of base are called abasic, either apurinic or apyrimidinic (AP). Alternatively, AP sites can be derived from spontaneous hydrolysis of the *N*-glycosyl bond. The AP site can be then eliminated in either of two ways: (i) an exonuclease or phosphodiesterase can act on the 5′-terminal desoxyribose-phosphate residue or (ii) bifunctional *N*-glycosylases/AP lyases may cut at the 3′ site. Repair of DNA is then carried out by the combined action of a DNA polymerase and a DNA ligase.

DNA glycosylases are widely distributed in nature. They were first studied in bacteria and named according to the modified "wrong" base they remove (see above). Five DNA glycosylases have been found in *S. cerevisiae*. They are encoded by *UNG1*, *OGG1*, *NGT1*, *NTG2*, and *MAG1* and remove uracil, oxidized and ring-opened purines, ring-saturated or fragmented pyrimidines and 8-oxoG, ring-saturated or fragmented pyrimidines, and methylpurines, respectively. Removal of damaged or modified bases leaves AP sites which are then cleaved at the 5′ side by AP endonucleases (Anp1 and Anp2) or at the 3′ side by AP lyases (Ntg1, Ntg2, and Ogg1). Anp1 is the main AP endonuclease, but its inactivation does not result in a detectable increase in genetic instability, although *anp1* mutants were moderately sensitive to methyl methanesulfonate (MMS) and oxidizing agents. However, an *anp1 anp2* double mutant is very sensitive to MMS, phleomycin, and H_2O_2. Therefore, in these assays Anp1 and Anp2 are redundant. In addition, three DNA glycosylases, those encoded by *NTG1*, *NTG2*, and *OGG1*, are also endowed with an AP lyase activity. They differ in cellular location and the sites they cleave (for a review, see reference 20).

Three BER genes have been characterized in *C. albicans*: *ANP1*, *OGG1*, and *NTG1* (90). Anp1 and Ogg1 are the orthologues of their *S. cerevisiae* counterparts, whereas Ntg1 is similar to both ScNtg1 and ScNtg2. Single null homozygous mutants in each of these genes exhibited normal growth and morphogenesis. Furthermore, they showed wild-type resistance to UV light and oxidizing agents (hydrogen peroxide and tetrabutyl hydrogen peroxide [TBHP]), suggesting that, as described for *S. cerevisiae*, these genes are redundant, and multiple disruptions are needed to observe any increase in the resistance to radiomimetic compounds like MMS. In this regard, even an *ntg1*-ΔΔ *ogg1*-ΔΔ double mutant was not more sensitive to oxidizing agents (2 mM H_2O_2 or 4 mM TBHP) than the parental strain. Importantly, *C. albicans* has been reported to be more resistant than *S. cerevisiae* to oxidizing agents, and this phenotype was extended to the respective BER mutants. It was suggested that *C. albicans* has evolved additional DNA repair systems to defend itself against killing by the oxygen radicals generated by macrophages. However, *ntg1* and *anp1* mutants survived phagocytosis by macrophages as much as their parental strain, and their fitness was not apparently affected in that process (92). It is likely that redundancy of the BER system applies also to *C. albicans*. It should be noticed, however, that in another study, *C. albicans* displayed sensitivities to H_2O_2 (5 mM), menadione (0.1 M), and TBHP (2 mM) similar to those of *S. cerevisiae* (59), suggesting that the genetic background may *cause* susceptibility. The frequency of LOH, determined by using the *GAL1/gal1*::*URA3* system, in *ntg1*, *apn1*, *apn1 ntg1*, and *ogg1 ntg1* mutants was also similar to that in wild-type cells. An analysis of selected SNP markers located at both sites of the *GAL1* marker (Fig. 1B) allowed inference of the mechanisms involved in LOH. Interestingly, *ogg1* mutants showed a wild-type LOH frequency for 2-DG^{R} strains but only 1/10 of the wild-type frequency for FOA^{R} clones. This suggests that the two homologues of Chr1 undergo unequal rates of LOH in the *ogg1* background. In all the cases, LOH was accompanied by homozygosis of the telomere-proximal SNP marker, suggesting that it resulted from either reciprocal CO or BIR. Resistance to fluconazole was not affected in these mutants (90). Other uncharacterized putative BER proteins, such as the bifunctional glycosylase uracil DNA glycosylase Udg, two AP endonucleases, the monofunctional glycosylase AlkA (which removes alkylated bases), and a glycosylase belonging to the Fpg/Nei family (CalFpg), are also present in *C. albicans*;

the last has been recently characterized in terms of substrate specificity (84).

NER

NER acts on lesions that distort the double helix, the most common being pyrimidine dimers caused by UV light. This recovery is independent from any photoreactivation (dark repair) and involves the repair of DNA containing those lesions. NER is a complex process that involves several steps. Following (i) recognition of the base damage, (ii) NER endonucleases cut the damaged DNA strand several nucleotides away from both 5′ and 3′ sides of the pyrimidine dimer; (iii) a helicase uncoils the doubly cleaved (damaged) strand, removing about 25 nucleotides. The gap is then (iv) filled by polymerases and (v) sealed by the action of a ligase. The pathway has been best studied for bacteria. The two first steps are carried out by three proteins (UvrA, UvrB, and UvrC) acting in a sequential manner, although the complex is usually referred to as UvrABC endonuclease. Release of the oligonucleotide fragment is carried out by the action of the helicase UvrD. In the final step, DNA polymerase I and DNA ligase carry out the repair synthesis reactions (reviewed in reference188).

Three epistasis groups of genes (*RAD3*, *RAD52*, and *RAD6*) are involved in the recovery of *S. cerevisiae* cells from UV and/or ionizing radiation. Most mutants from the *RAD3* group are very sensitive to UV radiation and many chemicals because they are defective in NER. This group includes many genes (*RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD7*, *RAD10*, *RAD14*, *SSL1*, *SSL2/RAD25*, *TFB1*, *RAD16*, *RAD23*, and *MMS19*), but sensitivity to UV light varies among gene deletion mutants. Many of them are involved in steps that follow oligonucleotide excision (i.e., in synthesis of DNA and ligation) or in some other essential cellular function (i.e., *RAD3* and *RAD25* are components of the TFIIH complex involved in transcription) and therefore are not NER specific. Others, such as *RAD1*, *RAD2*, and *RAD10*, are highly specific, and their inactivation results in a total defect in NER. Rad1 and Rad10 form a complex endowed with endonuclease activity, which cleaves 5′ to sites of base damage. Rad2 has a polarity opposite of Rad1-Rad10 and cleaves 3′ to sites of base damage during NER (66, 145). The Rad1-Rad10 complex is involved in a variety of functions in *S. cerevisiae*, including BER and recombinational repair through synthesis-dependent strand annealing (SDSA) and single-strand annealing (SSA). In the BER pathway, it acts as a backup enzyme for repair of AP sites by cleaving the 3′ sugar phosphate because of its flap endonuclease activity. In SDSA, Rad1-Rad10 may act when the newly repaired strand differs in sequence from the original sequence, and in SSA, in combination with the MMR proteins Msh2-Msh3, it cuts the 3′ overhanging single strands left by the exonuclease digestion of the DNA ends produced by a DSB (101). Finally, the Rad1-Rad10 endonuclease has been implicated in the generation of translocations, large deletions, and de novo telomere addition (73).

RAD2 and *RAD10* have been characterized in *C. albicans*. Mutants with deletions in either or both genes are extremely sensitive to UV light. By comparison, the same mutants from *S. cerevisiae* are less sensitive and recover better from UV damage. It has been suggested that *S. cerevisiae* has evolved additional pathways to protect itself from UV damage, which is consistent with its way of life on fruits and vegetables exposed to direct sunlight. One of these pathways likely involves a photolyase, which, as noted above, is absent in *C. albicans*. The same deletion mutants did not show an alteration of the wild-type LOH when examined using the *GAL1/URA3* system described above (91).

Repair of Double-Strand Breaks (DSBs)

HR

In *S. cerevisiae*, two major mechanisms of conservative HR have been described: DSBR, which may result in GC with or without CO, and SDSA, a source of GC without CO. HR uses proteins of the Rad52 epistasis group (Rad51, Rad52, Rad54, Rad55/57, and Rad59) and the Mre11/Rad50/Xrs2 complex (MRX complex), helped by Sae2, and the exonucleases Exo1 and Sgs1-Dna2, which are involved in the resection of the DSB ends (for reviews, see references 86, 97, 114, 115, 135, and 184). A model that takes into account recent observations on the molecular bases of both mechanisms is shown in Color Plate 1. Rad51 plays a central role in the homology search and catalyzes strand exchange between the recombination partners (169). Rad52 catalyzes the exchange of replication protein A (RPA) for Rad51 on single-stranded DNA (ssDNA) (mediator function) (180), and later it promotes the second-end capture, owing to its annealing activity (126). In *S. cerevisiae*, the *RAD51*-dependent pathway of recombination is used preferentially in interchromosomal transactions, such as GC, and is also required for repair of most DSBs in mitotic cells (143, 184). In some cases, a one-ended DSB may occur because of replication fork collapse or erosion of uncapped telomeres (114) (or two-ended DSB, but only one has homology to the donor molecule [107, 119]). These lesions can be still repaired by BIR, a process in which the 3′ end of the DSB invades a homologous DNA duplex, forming a replication fork that copies sequences *to* the end of the molecule (106, 119). As with COs, BIR results in extensive LOH. Since band invasion is required for GC, GC plus CO, and most BIR events, these events are dependent on Rad51 (and the other proteins of the Rad52 epistasis group). Most Rad51-dependent BIR events are also dependent on Pol32, a subunit of the replicating polymerase complex polymerase δ, whose deletion results in half-COs (40, 99, 171). BIR can cause GCR if one end (or both) of the resected DSB exposes a highly repeated genome element, like the Ty retrotransposon. This end can invade one copy of the repeated element at another site (i.e., a nonhomologous chromosome) and repair the break using BIR(27, 147).

Some Rad51-independent events have been described to occur in *S. cerevisiae*. Rad51 is dispensable, if not inhibitory, for SSA, a Rad52-dependent HR mechanism that promotes recombination between direct repeats with the loss of one repeat and the intervening sequence (Color Plate 1) (76, 113, 178). In *S. cerevisiae*, Rad59, a Rad52 paralogue, is thought to function as an accessory factor that augments the SSA activity of Rad52 (15, 16, 39). In fact, overexpression of Rad52 suppresses the *rad59* defect (16). Rad59 also cooperates to support the machinery required for cleavage of nonhomologous tails, which is carried out by the NER endonuclease Rad1-Rad10 (134). Consistent with the annealing activity of Rad59, the *rad59* mutant exhibits decreased efficiency in SSA, especially when the direct repeats are short (39, 133, 179). Also, spontaneous recombination between chromosomal inverted repeats is dependent on Rad52 but largely independent of Rad51, and the Rad51-independent recombination is dependent on Rad59 (16, 148). Recent results suggest that in the inverted-repeat system,

spontaneous Rad51-independent recombination occurs not by strand invasion but by an SSA mechanism during DNA synthesis, which explains the requirement for Rad52 and Rad59 (122). As described for BIR, when occurring between repetitive elements located on different chromosomes, SSA can cause GCR (27). A Rad51-independent repair pathway of chromosomal DSB with the physical characteristics of BIR also seems to occur in two different situations: cleavage at MAT in one chromosome III homologue and in the maintenance of telomeres in the absence of telomerase. This pathway requires Rad52, Rad59, Tid1, and the MRX complex (16, 40, 106, 107) and has been suggested to occur by BIR followed by SSA (75). Finally, spontaneous GC events with or without COs may also occur at a low frequency in the absence of Rad51 or Rad52 but not both proteins (33, 140).

RAD51, *RAD52*, *RAD59*, *RAD50*, and *MRX11* have been characterized in *C. albicans*. Rad52 was shown to be crucial for DNA repair, gene replacement (gene targeting), and virulence in a murine model of hematogenously disseminated candidiasis (28, 32, 59). Among the null homozygous mutants in HR genes, the *rad52-ΔΔ* mutant was by far the most sensitive to DNA-damaging agents, including UV light, MMS, the antitumor compounds bleomycin and camptothecin, and the oxidizing agents hydrogen peroxide, menadione, and TBHP. Null *rad51* mutants also displayed sensitivity, but they were significantly less affected. Rad59 was needed only when the damage caused by UV or MMS exceeded a threshold (59). Null *rad50* and *mre11* mutants displayed also significant sensitivity to TBHP, H_2O_2, and UV radiation (91). Notably, the three genes *RAD51*, *RAD52*, and *RAD59* showed haploinsufficiency for UV repair, suggesting that HR interacts with the NER pathway to remove pyrimidine dimers in *C. albicans*. In the absence of *RAD52*, spontaneous LOH increases significantly, but it occurs by chromosome loss and chromosome truncation, two highly mutagenic mechanisms that were not detected in wild-type cells, where GC with COs or BIR were responsible for all LOH events (6, 7, 91). LOH was also significantly increased in *mre11* and *rad50* null homozygous strains (91). In *rad51* deletion mutants, LOH occurred mainly by chromosome loss (53%) and chromosome truncation (20%), but GC or BIR events were also detected at a significant frequency (27%). In contrast to Rad52, Rad51 was also dispensable for gene targeting, even though the efficiency of the process was reduced to 20% (F. García-Prieto, unpublished results). Recombination between the hisG repeats present in the *his*G-*URA3*-*his*G module of the URA blaster cassettes, which occurred by SSA, was not affected in *rad51*, whereas the same process is highly defective in the *rad52* counterpart and leads to chromosome truncation at a frequency of 80% (6). Therefore, as shown for *S. cerevisiae*, the Rad52 gene is the most important gene involved in HR in *C. albicans*.

HR could also be involved in the generation of the isochromosome i(5L). This process likely takes advantage of inverted repeats that flank the centromere of Chr5 (18, 159) which allow repair by BIR following resection of a DSB in the right arm (see reference 163 for a model). The centromeric region of other chromosomes lacks those inverted repeats, and no isochromosomes derived from them have been reported so far.

Interestingly, disruption of both alleles of any of the several *C. albicans* genes required for efficient and accurate HR, including *RAD51*, *RAD52*, *RAD50*, and *MRE11*, results in both a decrease in growth rate and a polarization of growth that give rise to filaments formed by elongated pseudohyphal cells (8, 59, 91). Constitutive filamentation of *rad52* homozygous cells is accompanied by expression of hypha-specific genes, including *ECE1* and *HWP1*, but deletion of these genes or the G_1 cyclin *HGC1*, which is absolutely required for hyphal growth (192), does not prevent filamentation. Filamentation appears to be linked to uncoupling of several events of the cell cycle likely derived from the accumulation of unrepaired DSBs (8). Confirming this possibility, the extent of filamentation exhibited by the several mutants is proportional to the importance of each gene in the repair of DSB caused by several agents (59).

NHEJ

A second recombination pathway exists, NHEJ, that can rejoin the two ends of a DSB by simple ligation after little or no nucleolytic processing of the end. In budding yeast, the Yku70/Yku80 complex and Lig4 and its associated Lif1/Nej1 complex, as well as the MRX complex, are required for NHEJ (38). Among the homologues of these genes in *C. albicans*, only *LIG4* and *KU80* have been partially characterized (10, 91). In *S. cerevisiae*, NHEJ is repressed in diploids where the HR pathway is active. This is presumably advantageous because diploid cells most often contain one intact copy of each gene for the repair of damaged DNA through HR, a high-fidelity repair mechanism. Therefore, the retention of NHEJ genes in an obligate diploid such as *C. albicans* suggests additional important roles. In fact, besides its role in end joining, the Ku proteins have been implicated in a variety of functions at telomeres (49, 50, 176), and the same is true for *C. albicans* (our unpublished results). Lig4 played a minor role in the repair of DNA damage (only after severe DNA damage were *lig4* deletion mutants more sensitive to UV light and MMS than the wild type). Its deletion did not affect chromosome stability but resulted in a slight to moderate attenuation of virulence in a murine model of candidiasis (9, 10, 32).

TLS

In spite of the several mechanisms that avoid or remove DNA lesions, not all of them are repaired. Unrepaired lesions can block the progression of the replication fork because regular polymerases stop at the site of lesions. Both prokaryotes and eukaryotes have evolved polymerases that bypass the lesion despite the lack of information or the misinformation present in the damaged template DNA. This allows replication to continue but at the cost of introducing mutations in the newly synthesized DNA. The DNA polymerases involved are small and have relaxed shape-based mechanisms and very low processivity, in such a way that they are replaced by regular polymerases once the lesion is bypassed (144). Among the lesions that can be bypassed by these polymerases are those caused by mutagenic agents (i.e., thymine dimers), but also abasic AP sites that form frequently as a consequence of the spontaneous hydrolysis of bases from DNA. In bacteria, members of the Y family of translesion DNA polymerases have been indentified as responsible for the processing of DNA damage to mutations during the SOS response, including UmuDC (polymerase IV), DinB (polymerase IV), and MucAB (polymerase R1). Mutants with changes in these genes are defective in the generation of SOS-induced mutagenesis. Five TLS polymerases have been described for *S. cerevisiae*: *REV1*, *REV3*, *REV7*, *RAD30*, and *POL32*. Rev1 inserts

dCMP residues opposite of an AP lesion (124) but also helps to bypass some UV light-induced photoproducts using a mechanism other than its transferase activity (89). Rev3 and Rev7 form a heterodimer known as polymerase ξ, a weakly processive polymerase involved in general TLS (48). Rad50/polymerase η is used in the error-free bypass of thymine dimers and oxo-G. Mutations of this polymerase in humans cause xeroderma pigmentosum variant syndrome (111). Polymerase 32 is a subunit of polymerase δ that, in addition to its role in BIR, has several poorly understood functions. Among the phenotypes of the *pol32* mutants of *S. cerevisiae* are sensitivity to DNA-damaging agents, cold sensitivity, and an increase in both HR and genetic instability (60, 67). Deletion of *POL32* caused a rather weak deficit in background mutagenesis; however, when accompanied by induction of mutator DNA glycosylases that generate an excess of AP sites it resulted in cell death, suggesting a crucial role for polymerase 32 in bypassing abasic sites during TLS (14). Homologues of these proteins have been found but not yet characterized in *C. albicans*. Because of their role in survival under stress conditions, they may be important in the adaptation of *C. albicans* to the conditions the organism encounters during infection.

In *S. cerevisiae*, other genes are also involved in TLS, although they may have additional activities. Rad6 is an E2-ubiquitin conjugating enzyme that uses three different E3 ubiquitin ligases—Rad18, Bre1, and Ubr1—to ubiquitinate different substrates, including the DNA polymerase sliding clamp PCNA and histone H2B, which, in turn, affect TLS and error-free repair (37, 71; reviewed in reference 58). The *RAD6* orthologue has been characterized in *C. albicans*. The protein showed a high degree of sequence similarity to Rad6 proteins from fungi to humans, and Ca*RAD6* complemented an *S. cerevisiae rad6* null mutation, suggesting that the proteins have similar functions in both yeasts. However, in *C. albicans*, *RAD6* could be essential, since the double knockout could not be generated. As expected from its role in DNA repair, depletion of Ca*Rad6* caused UV sensitivity and RAD6 expression was induced by UV light. Importantly, Rad6 depletion enhanced pseudohypal growth (92). This could be a consequence of the participation of Rad6 in a Rad51-dependent HR pathway, and a reminiscence of the filamentation observed in other recombination mutants from the Rad52 epistasis group.

MECHANISMS OF DRUG RESISTANCE DERIVED FROM GENETIC INSTABILITY

For an opportunistic pathogen, drug resistance represents an excellent and practical system to correlate phenotypic traits with genomic changes. Azoles are drugs commonly used in clinics. However, as expected from their fungistatic activity, they frequently induce resistance. Several mechanisms of resistance to azoles are reasonably well understood. The target of azoles is the *ERG11* gene product, a lanosterol demethylase involved in the biosynthesis of ergosterol from squalene. Inhibition of that activity causes the production of toxic sterols that accumulate in the membrane of *C. albicans*. Resistance to azoles may occur by several mechanisms, including (i) chemical alteration of the target, (ii) an increase in the amount of target molecules, and (iii) a drop in the drug concentration inside the cell. Azoles are excreted from cells through two types of efflux pumps: ABC transporters, which use the energy of ATP and are represented by Cdr1 and Cdr2, and MFS (major facilitator superfamily) transporters, which are driven by proton energy and are represented by Mdr1. Cdr1 exports fluconazole, ketozonazole, and voriconazole and is the cause of most resistance in *C. albicans*. Mdr1 exports preferentially fluconazole (and, to a lesser extent, voriconazole) and is involved in most resistance in the closely related species *C. dubliniensis*. For additional information on drug resistance see chapters 23 and 25, as well as recent reviews (120, 158). Although they occur at a lower frequency, resistance to echinocandins has been reported to occur in clinical isolates and laboratory strains. This resistance has been traced to amino acid substitutions in two hot-spot regions of the glucan synthase catalytic subunit *GSC1/FLK1* (for a review, see reference 139).

Genetic Changes That Cause Alterations in the Target

ERG11 carries numerous nonsynonymous polymorphisms. In fact, more than 110 protein variants have been reported for azole-resistant strains (158), although only a few have been unambiguously associated with azole resistance (118). This is a reflection of the high permissiveness of the molecule to changes in sequence and suggests an elevated rate of point mutation in *C. albicans*. The importance of each substitution in resistance is difficult to define, since changes usually occur in groups of two to four. Still, some changes appear more frequently in resistant strains. Interestingly, *ERG11* from *C. dubliniensis* is 96% identical to Ca*ERG11*, and mutations in ERG11 have been also found in azole-resistant strains of *C. dubliniensis*; some of them are common to those found in resistant counterparts from *C. albicans*, but others are different (137). A characteristic of the fluconazole resistance associated with *ERG11* alterations is that both alleles are in homozygosis (189) (Color Plate 2). This circumstance, together with the localization of *ERG11* near the *MTL* locus in Chr5, served to establish a correlation between the homozygosity at the *MTL* and the acquisition of resistance to azoles (149). *MTL* homozygous strains frequently have lost one copy of Chr5 and have duplicated the remaining homologue. Therefore, all genes located in that chromosome, including *ERG11* and *TAC1* (see below), have become homozygous.

A recent study aimed at determining mechanisms of echinocandin resistance has identified an S645P mutation in one of the *GSC1* alleles of a micafungin-resistant clinical isolate. LOH resulting in two alleles carrying the S645P mutation was required to acquire high-level echinocandin resistance. Similarly, a derivative from a laboratory strain selected for reduced sensitivity to micafungin had a single heterozygous amino acid change (S645/S645F) in *GSC1*, whereas no mutations were found in the *S. cerevisiae FKS2* homologue Ca*GSL2/FKS2* or in Ca*RHO1*, which encodes the regulatory subunit of glucan synthase. Isogenic derivatives carrying combinations of resistant and sensitive alleles also indicate that a single base mutation followed by LOH was required for high levels of resistance to the echinocandins micafungin and caspofungin. Therefore, the *GSC1*-resistant allele seems to be semidominant, which is compatible with the additional finding that the two alleles contribute equally and independently to glucan synthase activity (125).

In summary, two manifestations of the genetic instability of *C. albicans*, point mutation and HR, contribute to the development of azole and echinocandin resistance in *C. albicans*.

Genetic Changes That Cause an Increase in the Amount of Target Molecules

Changes in the amount of Erg11p can be caused by an overexpression or an increase in the number of copies of *ERG11*. In *S. cerevisiae*, *ERG11* is regulated by two members of the Zn2Cys6 family, Sc*UPC2* and Sc*ECM22*. Only Ca*UPC2* has been found in the genome of *C. albicans*. It has homology to both genes present in *S. cerevisae*. It is known that *ERG11* is overexpressed in response to azoles and that the process is mediated by the transcriptional activator Upc2 (Color Plate 2). Deletion of *UPC2* eliminated overexpression of *ERG11* in response to azoles and drug resistance. In agreement with these observations, there is a conserved site for Upc2 binding in the promoter sequence of *ERG11* of *S. cerevisiae* and *C. albicans* which is also important for azole induction of *ERG11* overexpression (131, 170). As shown above for *ERG11*, an increase in the activity of Upc2 due to changes in its amino acid sequence followed by homozygosity resulted in an increase in azole resistance (46, 69). However, there are other mechanisms for azole induction of *ERG11* that remain to be investigated.

Genetic Changes That Result in a Decrease in Azole Concentration in the Cell

An increase in azole export rate decreases the drug concentration inside the cell. This can be achieved by increasing the amount or the activity of the efflux pumps Cdr1, Cdr2, and Mdr1 (Color Plate 2).

The ABC transporters Cdr1 and Cdr2 are regulated by the transcriptional activator Tac1, and *TAC1* deletion downregulates expression of *CDR1* and *CDR2*. *TAC1* is located on the left arm of Chr5, whereas *CDR1* and *CDR2* are on the right arm of Chr3. Tac1 binds to the *cis*-acting region DRE (drug-responsive element) present in the promoters of *CDR1* and *CDR2*. Several mutations in the activation domain of Tac1 are associated with an increase in fluconazole resistance, and the corresponding *TAC1* alleles are known as hyperactive alleles. Notably, the hyperactive allele is codominant with the wild type allele, in such a way that the former does not induce CDR overexpression when present in heterozygosis (Color Plate 2). Therefore, two copies of the hyperactive allele are needed to confer resistance to fluconazole, a process that may be achieved through LOH. As described for *ERG11*, many alleles of *TAC1* have been found, indicating, again, a high permissibility of the molecule to undergo changes in its primary sequence (34, 35). The location of *TAC1* on the left arm of Chr5 may also explain the correlation between *MTL* homozygosity and azole resistance (149).

The other efflux pump, Mdr1, belongs to the MFS family and is energized by the proton gradient. Two *cis*-acting elements, recognized by transcription factors Mcm1 and Mrr1, have been found in the promoter of *MDR1*. Elimination of Mcm1 does not affect azole resistance, whereas inactivation of Mrr1 in fluconazole-resistant strains renders them azole sensitive. *MDR1* is located on Chr6, whereas *MRR1* is on the right arm of Chr3. *C. albicans* strains whose azole resistance was caused by overexpression of *MDR1* carry modified alleles of *MRR1*, and these alleles conferred resistance to several drugs when expressed in susceptible strains; furthermore, resistance was due to activation of *MDR1*, although other genes were possibly activated. Importantly, as mentioned for *TAC1*, in most of those resistant strains, both alleles of *MRR1* were in homozygosis, again indicating that single base mutation and LOH underlie azole resistance (45, 121, 162) (Color Plate 2).

Genetic Changes in the Copy Number of Genes Involved in Azole Resistance

Azole resistance in vitro and in vivo is frequently associated with changes in chromosome copy number, segmental aneuploidies, and general aneuploidy (36, 109, 165). In *C. albicans*, aneuploidies accounted for 50% of 70 fluconazole-resistant strains, whereas they were found in only 10% of nonresistant counterparts. Of the aneuploid strains, 20% of them carried the isochromosome i(5L) formed by two left arms of Chr5, other aneuploidies being less represented (<10%). Furthermore, loss of i(5L) resulted in a loss of fluconazole resistance (164, 165). As noticed, formation of i(5L) results in duplication of *ERG11* and *TAC1*.

A recent report has analyzed karyotypic changes that evolved when populations of *C. albicans* were subjected to increasing concentrations of fluconazole over many generations (164). In response to the selective pressure, the MICs of fluconazole for three different populations increased significantly along with, in parallel, significant changes in their karyotypes. By contrast, populations not exposed to the drug remained fluconazole sensitive and conserved their initial karyotypes. A high percentage of the isolates obtained at different times from the three populations maintained in fluconazole showed the isochromosome i(5L), in one or multiple copies. Other isolates showed additional chromosomal bands that represented new aneuploidies. For instance, one isolate carried sequences of Chr5L in four different chromosomes: two copies of intact Chr5, one i(5L), and one chimerical chromosome characterized as i(5L) bound to a fragment of Chr3R [i(5L)-3R]. In this extra chromosome, the Chr3R breakpoint was just to the left of *CDR1*, and therefore it also included *MRR1*. Another isolate carried an extra band characterized as an i(5L) attached to a regular Chr5. This structure, dicentric in nature, seemed to behave as a regular chromosome because of the functional inactivation of a centromere. However, it was unstable and was regularly lost. In general, the number of extra copies of Chr5L correlated with an increased fluconazole resistance (167). The nature and the dynamics of these GCR are not frequently found among yeasts, although similar changes, including chromosome fusion, occur at a high frequency in the haploid asexual *C. glabrata* and are connected also with antifungal drug resistance. Indeed, karyotypes from clinical isolates of *C. glabrata* have been shown to vary a lot (96, 141). Therefore, analysis of the junction sequences and an investigation of the pathways involved in their generation may reveal new targets for antifungal drugs. In fact, *C. albicans* mutants deficient in HR (*rad52*-ΔΔ, *mre11*-ΔΔ, and *rad50*-ΔΔ mutants) are more sensitive to fluconazole than wild-type cells (91). Recent studies with *S. cerevisiae* have demonstrated that suppressing different types of GCR involves different genetic requirements. In particular, formation and suppression of GCR mediated by nonallelic HR between divergent sequences use genes and mechanisms different from those used by GCR between single-copy sequences (147). Given the different kinds of repeats with identical or divergent sequences present in the *C. albicans* genome (i.e., ribosomal DNA repeats, MRS, telomeres, subtelomere repeats, retrotransoposons, and gene families) (105), rearrangements mediated by nonallelic HR between these repeats are expected to occur. They are likely counteracted by mechanisms that prevent an excess of these kinds of rearrangements.

In a significant number of isolates from the in vitro-adapted populations, the Chr5L aneuploidy was accompa-

nied by trisomies of one or several chromosomes, including Chr3, -4, -6, and -7. These alterations make sense since three of these chromosomes carry genes involved in Flu^R. Chr3 carries *CDR1* and *MRR1*, whereas Chr6 carries *MDR1*. Chr4 carries *NCP1*, which encodes the NADPH-cytochrome P450 reductase, a cofactor of Erg11p during the 14α-demethylation step, as well as other genes involved in ergosterol biosynthesis (*ERG8*, *ERG26*, and *ERG251*) (Color Plate 2). The frequent trisomy of Chr7 cannot be explained by the presence of genes involved in fluconazole resistance but could provide a selective advantage under stress conditions, as suggested previously (167), perhaps by correcting the gene imbalance caused by the other alterations. *C. albicans* has been proposed as a model yeast for this kind of study, whose importance is further highlighted by the fact that human tumor cells that have become resistant to chemotherapeutic agents also carry aneuploidies and these aneuploidies do not have a high fitness cost (163).

SPONTANEOUS VERSUS INDUCED GENETIC INSTABILITY

We now consider two aspects of the genetic stability which apparently have different genetic bases. In proliferating cell populations, genetic instability occurs spontaneously (i.e., in the absence of an obvious selective condition), resulting in preadaptation of part of the population. Alternatively, a stress condition may increase genomic instability through an increase in the mutation rate, often via pathways different from those used under normal growth conditions (151). In either situation, under selective conditions, the better-adapted genotypes (adaptive mutations) will spread throughout the population and become dominant. Examples of both kinds of mutational process have been described for *C. albicans*.

Rustchenko has provided evidence indicating that some strains, such as 3153A, produce spontaneous morphological mutants, which when analyzed exhibited variable degrees of karyotype alterations. Furthermore, one-third of the mutants were unstable and produced additional colony morphologies which were always paralleled by new karyotype changes. These random chromosomal alterations occurred at a frequency between 10^{-4} and 10^{-2} (150). However, Forche et al. (51) did not detect karyotype alterations in strain AF7, an SC5314 derivative, during its propagation in vitro, although it underwent short-range LOH events. Therefore, it seems that these strains of *C. albicans* possess variable degrees of genetic instability. When the AF7 strain was passaged in vivo, it maintained a similar rate of short-range GC (LOH) events. In addition, as mentioned above, it underwent long-range LOH and karyotype changes, usually associated with altered colony phenotypes. Therefore, in vivo populations undergo higher rates of genomic rearrangements and phenotypic changes than do in vitro populations (51). It is likely that the strong and continuous selection pressure imposed by the internal medium in the host allows growth and survival of the *C. albicans* populations that can adapt to stress through genomic arrangements.These observations further support the notion that at least two mechanisms, one spontaneous (short-range LOH) and another stress dependent (long-range LOH plus chromosomal alterations), operate to produce genetic instability in *C. albicans*. Interestingly, in the studies by Rustchenko and coworkers, the frequency of chromosomal changes increased under depletion of nutrients or with reduction in temperature (150, 156), suggesting that both mechanisms, spontaneous and stress-induced mutations, were operating simultaneously in a strain that was already unstable. Unfortunately, no characterization of the new chromosomal bands present in those mutants using Southern blot, SNP analysis, or comparative genome hybridization could be carried out at that time.

The importance of stress in the generation of chromosomal changes that results in an increase in fitness has been illuminated by the above-mentioned studies on the in vitro evolution of *C. albicans* in response to fluconazole (167). Unexpectedly, i(5L) was detected very early during the exposure to fluconazole (24 h) in three different populations. Although it is not possible to dispute that a few cells in the initial population carried i(5L) (preadaptation) or that fluconazole acts as a mutagen, the most likely explanation is that fluconazole is a stress that induces alternative pathways of mutation (167), regardless of whether it acts also as a mutagen. Not only was i(5L) very frequently found in clones from these in vitro evolution experiments, but also its presence in the most resistant clones in three different populations indicates that it confers a strong selective advantage in fluconazole. Reproducible aneuploidies had been found before in *S. cerevisiae* populations adapted to nutrient limitation conditions. However, in the same studies, other genetic alterations that conferred less fitness were also isolated. It is likely that both fitness advantages provided by the different kinds of mutations/rearrangements and clonal interference between the several phenotypes during the early phase of adaptation determine the frequency of the several clones by the end of the experiment (65, 83). Similarly, Flu^R clones of *C. albicans* lacking i(5L) were found, albeit at a much lower frequency (150).

In bacteria, stress-induced *lac*-to-Lac^+ reversion also occurs through both spontaneous mutation and starvation-induced stress. For the second mechanism, both point mutagenesis and gene amplification have been observed. There is evidence indicating that point mutagenesis results from a switch of regular DNA polymerases to an error-prone mutagenic DNA polymerase during the repair of DSBs caused by stress (142). Thus, point mutagenesis is stimulated by the induction of DSB and requires the starvation or stationary-phase stress response controlled by the transcriptional activator RpoS, the SOS DNA damage response, the HR-DSBR proteins, and functional error-prone DNA polymerases (57). It is likely that similar processes take place in eukaryotes and that the complexity of their fragmented genomes allows the generation of more complex genomic rearrangements (65). Furthermore, given both the complex relationship between phenotype and genotype, especially when complex traits encoded by multiple loci are being analyzed (43, 47), and the extreme plasticity of the *C. albicans* genome (163), we should expect extensive and variable stress-induced genomic variation during its adaptation to stress. In fact, expected and unexpected genomic alterations were observed in *S. cerevisiae* during evolution experiments under nutrient-limited conditions (65, 83).

Studies with bacteria also suggest that stress-induced mutagenesis may be limited to a subpopulation of cells that becomes transiently hypermutable. Hypermutable subpopulations are characterized by the presence of secondary mutations unrelated to that selected, which are distributed throughout the genome. Some of them allow the subpopulation to explore new ways to adapt to the stress. It is suggested that these dynamics avoid the acquisition of the deleterious effect of induced mutagenesis by the whole population, which then can reassume growth when the stress conditions disappear (62). A model to explain the presence

of hypermutable cell populations during the stress-induced Lac$^+$ reversion contemplates a bistable cell population, in which some cells have acquired simultaneously double strand breakage/SOS induction and the RpoS-controlled stress response (62). The concept of a transient hypermutable subpopulation able to adapt would fit well in the dynamic commensal pathogen that dominates the life history of *C. albicans*. However, in this case the hypermutable population is likely represented by the subpopulation that migrates from the mucosal surface into the internal tissues or subepidermal cell layers during the infectious process (51). Under different environmental stresses, as those represented by antifungal drugs or the internal medium of the animal, this subpopulation generates genomic changes driven by induction of DSB at an accelerated rate, followed by repair using mutagenic pathways. Most of the population remains in the commensal state and adopts a "resting state" that allows it to conserve the whole heterozygotic reservoir and the standard chromosome complement and, accordingly, the potential to adapt by chromosomal rearrangements. In contrast, highly virulent strains repeatedly selected for virulence in the internal tissues acquire chromosomal rearrangements that decrease their fitness in the commensal environment, where they will be outcompeted by the normal population (51).

FINAL REMARKS

Tolerance of *C. albicans* and *C. glabrata* to GCR and aneuploidies that do not have a high fitness cost suggest that they represent an aspect of genetic instablity which could be used frequently in asexual fungi of medical importance as mechanisms of adaptive mutations, in particular when they face stress caused by antifungal drugs and/or the variable and harsh conditions prevalent in the human body. It is likely that organisms that undergo meiosis have not evolved the potential or the mechanisms that mediate these genomic alterations. As noted previously, chromosomal dynamics are not compatible with a regular sexual cycle in which chromosomal pairing is required for meiotic segregation, which, in turn, explains why meiosis has been apparently lost in "asexual" yeasts (127, 141, 163). An investigation of the genetic mechanisms used to generate GCR and aneuploidies may reveal new specific pathways of recombination and therefore new antifungal targets.

It should be noted that aneuploidies and GCR influence the copy number or expression level of a large number of genes. Similarly, long-range LOH events resulting from COs, BIR, or chromosome loss followed by reduplication modify the allelic state of all the genes included in large chromosome segments or a whole chromosome. Therefore, these events could be advantageous to acquire phenotypes that are caused or influenced by multiple loci. Recent studies with *S. cerevisiae* indicate that one particular mutation has different effects in different genetic backgrounds, especially when the trait considered is influenced by several loci (43, 47). Given the differences among *C. albicans* isolates, different clones could also respond with different genomic changes to a particular stress. The universal distribution of some isolates suggests that *C. albicans* has coevolved with *Homo sapiens* (128). In this scenario, some genomic alterations generated by specific recombination pathways may have been selected in the past for *C. albicans* to adapt and survive in the harsh conditions found in the human body; furthermore, the ability to undergo these rearrangements likely represents a high selective pressure to maintain the general configuration of the *C. albicans* genome, including its specific telomeric, centromeric, and MRS regions.

This work was supported by grant SAF2007-60810 from Ministerio de Ciencia y Tecnología and grant 3PR05A076 from Junta de Extremadura to G.L. F.G.P. was supported by a fellowship from Junta de Extremadura. G.L. and R.C. were also supported by an NIH-NIAID grant, AI051949. We thank Fátima García-Prieto (UEx) for preparing the figures.

REFERENCES

1. **Aguilera, A., S. Chavez, and F. Malagon.** 2000. Mitotic recombination in yeast: elements controlling its incidence. *Yeast* **16:**731–754.
2. **Aguilera, A., and B. Gomez-Gonzalez.** 2008. Genome instability: a mechanistic view of its causes and consequences. *Nat. Rev. Genet.* **9:**204–217.
3. **Ahmad, A., M. Kabir, A. Kravets, E. Andaluz, G. Larriba, and E. Rustchenko.** 2008. Chromosome instability and unusual features of some widely used strains of *Candida albicans*. *Yeast* **25:**433–448.
4. **Alby, K., D. Schaefer, and R. Bennett.** 2009. Homothallic and heterothallic mating in the opportunistic pathogen *Candida albicans*. *Nature* **460:**890–893.
5. **Alonso-Monge, R., F. Navarro-Garcia, E. Roman, A. I. Negredo, B. Eisman, C. Nombela, and J. Pla.** 2003. The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*. *Eukaryot. Cell* **2:**351–361.
6. **Andaluz, E., A. Bellido, J. Gómez-Raja, A. Selmecki, K. Bouchonville, R. Calderone, J. Berman, and G. Larriba.** 2011. Rad52 function prevents chromosome loss and truncation in *Candida albicans*. *Mol. Microbiol.* **79:**1462–1482.
7. **Andaluz, E., J. Gomez-Raja, B. Hermosa, T. Ciudad, E. Rustchenko, R. Calderone, and G. Larriba.** 2007. Loss and fragmentation of chromosome 5 are major events linked to the adaptation of *rad52-ΔΔ* strains of *Candida albicans* to sorbose. *Fungal Genet. Biol.* **44:**789–798.
8. **Andaluz, E., T. Ciudad, J. Gómez-Raja, R. Calderone, and G. Larriba.** 2006. Rad52 depletion in *Candida albicans* triggers both the DNA-damage checkpoint and filamentation accompanied by but independent of expression of hypha-specific genes. *Mol. Microbiol.* **59:**1452–1472.
9. **Andaluz, E., T. Ciudad, and G. Larriba.** 2002. An evaluation of the role of *LIG4* in genomic instability and adaptive mutagenesis in *Candida albicans*. *FEMS Yeast Res.* **2:**341–348.
10. **Andaluz, E., R. Calderone, G. Reyes, and G. Larriba.** 2001. Phenotypic analysis and virulence of *Candida albicans LIG4* mutants. *Infect. Immun.* **69:**137–147.
11. **Anderson, J. B., C. Sirjusingh, and N. Ricker.** 2004. Haploidy, diploidy and evolution of antifungal drug resistance in *Saccharomyces cerevisiae*. *Genetics* **168:**1915–1923.
12. **Arbour, M., E. Epp, H. Hogues, A. Sellam, C. Lacroix, J. Rauceo, A. Mitchell, M. Whiteway, and A. Nantel.** 2009. Widespread occurrence of chromosomal aneuploidy following the routine production of *Candida albicans* mutants. *FEMS Yeast Res.* **9:**1070–1077.
13. **Argueso, J. L., J. Westmoreland, P. A. Mieczkowski, M. Gawel, T. D. Petes, and M. A. Resnick.** 2008. Double-strand breaks associated with repetitive DNA can reshape the genome. *Proc. Natl. Acad. Sci. USA* **105:**11845–11850.
14. **Auerbach, P. A., and B. Demple.** 2010. Roles of Rev1, Pol ζ, Pol32 and Pol η in the bypass of chromosomal abasic sites in *Saccharomyces cerevisiae*. *Mutagenesis* **25:**63–69.
15. **Bai, Y., A. P. Davis, and L. S. Symington.** 1999. A novel allele of *RAD52* that causes severe DNA repair and recom-

bination deficiencies only in the absence of *RAD51* or *RAD59*. *Genetics* **153:**1117–1130.
16. **Bai, Y., and L.S. Symington.** 1996. A Rad52 homolog is required for RAD51-independent mitotic recombination in *Saccharomyces cerevisiae*. *Genes Dev.* **10:**2025–2037.
17. **Barton, R. C., and S. Scherer.** 1994. Induced chromosome rearrangements and morphologic variation in *Candida albicans*. *J. Bacteriol.* **176:**756–763.
18. **Baum, M., K. Sanyal, P. K. Mishra, N. Thaler, and J. Carbon.** 2006. Formation of functional centromeric chromatin is specified epigenetically in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **103:**14877–14882.
19. **Bennett, R. J., and A. D. Johnson.** 2003. Completion of a parasexual cycle in *Candida albicans* by induced chromosome loss in tetraploid strains. *EMBO J.* **22:**2505–2515.
20. **Boiteux, S., and M. Guillet.** 2004. Abasic sites in DNA: repair and biological consequences in *Saccharomyces cerevisiae*. *DNA Repair* **3:**1–12.
21. **Bouchonville, K., A. Forche, K. E. Tang, A. Selmecki, and J. Berman.** 2009. Aneuploid chromosomes are highly unstable during DNA transformation of *Candida albicans*. *Eukaryot. Cell* **8:**1554–1566.
22. **Bougnoux, M. E., D. Diogo, N. Francois, B. Sendid, S. Veirmeire, J. F. Colombel, C. Bouchier, H. Van Kruiningen, C. d'Enfert, and D. Poulain.** 2006. Multilocus sequence typing reveals intrafamilial transmission and microevolutions of *Candida albicans* isolates from the human digestive tract. *J. Clin. Microbiol.* **44:**1810–1820.
23. **Bougnoux, M. E., D. M. Aanensen, S. Morand, M. Theraud, B. G. Spratt, and C. d'Enfert.** 2004. Multilocus sequence typing of *Candida albicans*: strategies, data exchange and applications. *Infect. Genet. Evol.* **4:**243–252.
24. **Bougnoux, M. E., S. Morand, and C. d'Enfert.** 2002. Usefulness of multilocus sequence typing for characterization of clinical isolates of *Candida albicans*. *J. Clin. Microbiol.* **40:**1290–1297.
25. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J. L. Reedy, I. Agrafioti, M. B. Arnaud, S. Bates, A. J. Brown, S. Brunke, M. C. Costanzo, D. A. Fitzpatrick, P. W. de Groot, D. Harris, L. L. Hoyer, B. Hube, F. M. Klis, C. Kodira, N. Lennard, M. E. Logue, R. Martin, A. M. Neiman, E. Nikolaou, M. A. Quail, J. Quinn, M. C. Santos, F. F. Schmitzberger, G. Sherlock, P. Shah, K. A. Silverstein, M. S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M. P. Stumpf, P. E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N. A. Gow, M. C. Lorenz, B. W. Birren, M. Kellis, and C. A. Cuomo.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459:**657–662.
26. **Campos, P. R., and L. M. Wahl.** 2010. The adaptation rate of asexuals: deleterious mutations, clonal interference and population bottlenecks. *Evolution* **64:**1973–1983.
27. **Chan, J. E., and R. D. Kolodner.** 2011. A genetic and structural study of genome rearrangements mediated by high copy repeat Ty elements. *PLoS Genet.* **7:**e1002089.
28. **Chauhan, N., T. Ciudad, A. Rodriguez-Alejandre, G. Larriba, R. Calderone, and E. Andaluz.** 2005. Virulence and karyotype analyses of *rad52* mutants of *Candida albicans*: regeneration of a truncated chromosome of a reintegrant strain (*rad52/RAD52*) in the host. *Infect. Immun.* **73:**8069–8078.
29. **Chen, X., B. B. Magee, D. Dawson, P. T. Magee, and C. A. Kumamoto.** 2004. Chromosome 1 trisomy compromises the virulence of *Candida albicans*. *Mol. Microbiol.* **51:**551–565.
30. **Chibana, H., and P. T. Magee.** 2009. The enigma of the major repeat sequence of *Candida albicans*. *Future Microbiol.* **4:**171–179.
31. **Chu, W. S., B. B. Magee, and P. T. Magee.** 1993. Construction of an *Sfi*I macrorestriction map of the *Candida albicans* genome. *J. Bacteriol.* **175:**6637–6651.
32. **Ciudad, T., E. Andaluz, O. Steinberg-Neifach, N. F. Lue, N. A. Gow, R. A. Calderone, and G. Larriba.** 2004. Homologous recombination in *Candida albicans*: role of CaRad52p in DNA repair, integration of linear DNA fragments and telomere length. *Mol. Microbiol.* **53:**1177–1194.
33. **Cöic, E., T. Feldman, A. S. Landman, and J. E. Haber.** 2008. Mechanisms of Rad52-independent spontaneous and UV-induced mitotic recombination in *Saccharomyces cerevisiae*. *Genetics* **179:**199–211.
34. **Coste, A., A. Selmecki, A. Forche, D. Diogo, M. E. Bougnoux, C. d'Enfert, J. Berman, and D. Sanglard.** 2007. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. *Eukaryot. Cell* **6:**1889–1904.
35. **Coste, A., V. Turner, F. Ischer, J. Morschhauser, A. Forche, A. Selmecki, J. Berman, J. Bille, and D. Sanglard.** 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics* **172:**2139–2156.
36. **Cowen, L., D. Sanglard, D. Calabrese, C. Sirjusingh, J. B. Anderson, and L. M. Kohn.** 2000. Evolution of drug resistance in experimental populations of *Candida albicans*. *J. Bacteriol.* **182:**1515–1522.
37. **Daigaku, Y., A. A. Davies, and H. D. Ulrich.** 2010. Ubiquitin-dependent DNA damage bypass is separable from genome replication. *Nature* **465:**951–955.
38. **Daley, J. M., P. L. Palmbos, D. Wu, and T. E. Wilson.** 2005. Nonhomologous end joining in yeast. *Annu. Rev. Genet.* **39:**431–451.
39. **Davis, A. P., and L. S. Symington.** 2001. The yeast recombinational repair protein Rad59 interacts with Rad52 and stimulates single-strand annealing. *Genetics* **159:**515–525.
40. **Deem, A., K. Barker, K. Vanhulle, B. Downing, A. Vayl, and A. Malkova.** 2008. Defective break-induced replication leads to half-crossovers in *Saccharomyces cerevisiae*. *Genetics* **179:**1845–1860.
41. **Desai, M. M., D. S. Fisher, and A. W. Murray.** 2007. The speed of evolution and maintenance of variation in asexual populations. *Curr. Biol.* **17:**385–394.
42. **Diogo, D., C. Bouchier, C. d'Enfert, and M. E. Bougnoux.** 2009. Loss of heterozygosity in commensal isolates of the asexual diploid yeast *Candida albicans*. *Fungal Genet. Biol.* **46:**159–168.
43. **Dowell, R. D., O. Ryan, A. Jansen, D. Cheung, S. Agarwala, T. Danford, D. A. Bernstein, P. A. Rolfe, L. E. Heisler, B. Chin, C. Nislow, G. Giaever, P. C. Phillips, G. R. Fink, D. K. Gifford, and C. Boone.** 2010. Genotype to phenotype: a complex problem. *Science* **328:**469.
44. **Dunham, M. J., H. Badrane, T. Ferea, J. Adams, P. O. Brown, F. Rosenzweig, and D. Botstein.** 2002. Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **99:**16144–16149.
45. **Dunkel, N., J. Blass, P. D. Rogers, and J. Morschhauser.** 2008. Mutations in the multi-drug resistance regulator *MRR1*, followed by loss of heterozygosity, are the main cause of *MDR1* overexpression in fluconazole-resistant *Candida albicans* strains. *Mol. Microbiol.* **69:**827–840.
46. **Dunkel, N., T. T. Liu, K. S. Barker, R. Homayouni, J. Morschhauser, and P. D. Rogers.** 2008. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate. *Eukaryot. Cell* **7:**1180–1190.

47. **Ehrenreich, I. M., N. Torabi, Y. Jia, J. Kent, S. Martis, J. A. Shapiro, D. Gresham, A. A. Caudy, and L. Kruglyak.** 2010. Dissection of genetically complex traits with extremely large pools of yeast segregants. *Nature* **464:**1039–1042.
48. **Endo, K., Y. Tago, Y. Daigaku, and K. Yamamoto.** 2007. Error-free *RAD52* pathway and error-prone *REV3* pathway determines spontaneous mutagenesis in *Saccharomyces cerevisiae*. *Genes Genet. Syst.* **82:**35–42.
49. **Espejel, S., S. Franco, S. Rodriguez-Perales, S. D. Bouffler, J. C. Cigudosa, and M. A. Blasco.** 2002. Mammalian Ku86 mediates chromosomal fusions and apoptosis caused by critically short telomeres. *EMBO J.* **21:**2207–2219.
50. **Fisher, T. S., and V. A. Zakian.** 2005. Ku: a multifunctional protein involved in telomere maintenance. *DNA Repair* **4:**1215–1226.
51. **Forche, A., P. T. Magee, A. Selmecki, J. Berman, and G. May.** 2009. Evolution in *Candida albicans* populations during a single passage through a mouse host. *Genetics* **182:**799–811.
52. **Forche, A., K. Alby, D. Schaefer, A. D. Johnson, J. Berman, and R. J. Bennett.** 2008. The parasexual cycle in *Candida albicans* provides an alternative pathway to meiosis for the formation of recombinant strains. *PLoS Biol.* **6:**e110.
53. **Forche, A., G. May, and P. T. Magee.** 2005. Demonstration of loss of heterozygosity by single-nucleotide polymorphism microarray analysis and alterations in strain morphology in *Candida albicans* strains during infection. *Eukaryot. Cell* **4:**156–165.
54. **Forche, A., P. T. Magee, B. B. Magee, and G. May.** 2004. Genome-wide single-nucleotide polymorphism map for *Candida albicans*. *Eukaryot. Cell* **3:**705–714.
55. **Forche A., G. May, J. Beckerman, S. Kauffman, J. Becker, and P. T. Magee.** 2003. A system for studying genetic changes in *Candida albicans* during infection. *Fungal Genet. Biol.* **39:**38–50.
56. **Fradin, C., M. Kretschmar, T. Nichterlein, C. Gaillardin, C. d'Enfert, and B. Hube.** 2003. Stage-specific gene expression of *Candida albicans* in human blood. *Mol. Microbiol.* **47:**1523–1543.
57. **Galhardo, R. S., P. J. Hastings, and S. M. Rosenberg.** 2007. Mutation as a stress response and the regulation of evolvability. *Crit. Rev. Biochem. Mol. Biol.* **42:**399–435.
58. **Game, J. C., and S. B. Chernikova.** 2009. The role of *RAD6* in recombinational repair, checkpoints, and meiosis via histone modification. *DNA Repair* **9:**470–482.
59. **Garcia-Prieto, F., J. Gomez-Raja, E. Andaluz, R. Calderone, and G. Larriba.** 2010. Role of the homologous recombination genes *RAD51* and *RAD59* in the resistance of *Candida albicans* to UV light, radiomimetic and anti-tumor compounds and oxidizing agents. *Fungal Genet. Biol.* **47:**433–445.
60. **Gerik, K. J., X. Li, A. Pautz, and P. M. Burgers.** 1998. Characterization of the two small subunits of *Saccharomyces cerevisiae* DNA polymerase delta. *J. Biol. Chem.* **273:**19747–19755.
61. **Gomez-Raja, J., E. Andaluz, B. Magee, R. Calderone, and G. Larriba.** 2008. A single SNP, G929T (Gly310Val), determines the presence of a functional and a nonfunctional allele of HIS4 in *Candida albicans* SC5314: detection of the non-functional allele in laboratory strains. *Fungal Genet. Biol.* **45:**527–541.
62. **Gonzalez, C., L. Hadany, R. G. Ponder, M. Price, P. J. Hastings, and S. M. Rosenberg.** 2008. Mutability and importance of a hypermutable cell subpopulation that produces stress-induced mutants in *Escherichia coli*. *PLoS Genet.* **4:**e1000208.
63. **Graser, Y., M. Volovsek, J. Arrington, G. Schonian, W. Presber, T. G. Mitchell, and R. Vilgalys.** 1996. Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. *Proc. Natl. Acad. Sci. USA* **93:**12473–12477.
64. **Greenberg, J. R., N. P. Price, R. P. Oliver, F. Sherman, and E. Rustchenko.** 2005. *Candida albicans SOU1* encodes a sorbose reductase required for L-sorbose utilization. *Yeast* **22:**957–969.
65. **Gresham, D., M. M. Desai, C. M. Tucker, H. T. Jenq, D. A. Pai, A. Ward, C. G. DeSevo, D. Botstein, and M. J. Dunham.** 2008. The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS Genet.* **4:**e1000303.
66. **Habraken, Y., P. Sung, L. Prakash, and S. Prakash.** 1995. Structure-specific nuclease activity in yeast nucleotide excision repair protein Rad2. *J. Biol. Chem.* **270:**30194–30198.
67. **Hanna, M., L. G. Ball, A. H. Tong, C. Boone, and W. Xiao.** 2007. Pol32 is required for Pol zeta-dependent translesion synthesis and prevents double-strand breaks at the replication fork. *Mutat. Res.* **625:**164–176.
68. **Harfe, B. D., and S. Jinks-Robertson.** 2000. Mismatch repair proteins and mitotic genome stability. *Mutat. Res.* **451:**151–167.
69. **Heilmann, C. J., S. Schneider, K. S. Barker, P. D. Rogers, and J. Morschhauser.** 2010. An A643T mutation in the transcription factor Upc2p causes constitutive *ERG11* upregulation and increased fluconazole resistance in *Candida albicans*. *Antimicrob. Agents Chemother.* **54:**353–359.
70. **Hicks, W. M., M. Kim, and J. E. Haber.** 2010. Increased mutagenesis and unique mutation signature associated with mitotic gene conversion. *Science* **329:**82–85.
71. **Hoege, C., B. Pfander, G. L. Moldovan, G. Pyrowolakis, and S. Jentsch.** 2002. *RAD6*-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419:**135–141.
72. **Hube, B.** 2004. From commensal to pathogen: stage- and tissue-specific gene expression of *Candida albicans*. *Curr. Opin. Microbiol.* **7:**336–341.
73. **Hwang, J. Y., S. Smith, and K. Myung.** 2005. The Rad1-Rad10 complex promotes the production of gross chromosomal rearrangements from spontaneous DNA damage in *Saccharomyces cerevisiae*. *Genetics* **169:**1927–1937.
74. **Ibrahim, A. S., B. B. Magee, C. D. Sheppard, M. Yang, S. Kauffman, J. Becker, J. E. Edwards, Jr., and P. T. Magee.** 2005. Effects of ploidy and mating type on virulence of *Candida albicans*. *Infect. Immun.* **73:**7366–7374.
75. **Ira, G., and J. E. Haber.** 2002. Characterization of *RAD51*-independent break-induced replication that acts preferentially with short homologous sequences. *Mol. Cell. Biol.* **22:**6384–6392.
76. **Ivanov, E. L., N. Sugawara, K. Fishman-Lobell, and J. E. Haber.** 1996. Genetics requirements for the single-strand break annealing pathway of double strand break repair in *Saccharomyces cerevisiae*. *Genetics* **142:**693–704.
77. **Iwaguchi, S. I., M. Sato, B. B. Magee, P. T. Magee, K. Makimura, and T. Suzuki.** 2001. Extensive chromosome translocation in a clinical isolate showing the distinctive carbohydrate assimilation profile from a candidiasis patient. *Yeast* **18:**1035–1046.
78. **Iwaguchi, S. I., M. Homma, and K. Tanaka.** 1990. Variation in the electrophoretic karyotype analysed by the assignment of DNA probes in *Candida albicans*. *J. Gen. Microbiol.* **136:**2433–2442.
79. **Janbon, G., F. Sherman, and E. Rustchenko.** 1998. Monosomy of a specific chromosome determines L-sorbose utilization: a novel regulatory mechanism in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **95:**5150–5155.
80. **Jiricny, J.** 2006. The multifaceted mismatch-repair system. *Nat. Rev. Mol. Cell Biol.* **7:**335–346.

81. **Jones, T., N. A. Federspiel, H. Chibana, J. Dungan, S. Kalman, B. B. Magee, G. Newport, Y. R. Thorstenson, N. Agabian, P. T. Magee, R. W. Davis, and S. Scherer.** 2004. The diploid genome sequence of *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **101:**7329–7334.
82. **Kabir, M. A., A. Ahmad, J. R. Greenberg, Y. K. Wang, and E. Rustchenko.** 2005. Loss and gain of chromosome 5 controls growth of *Candida albicans* on sorbose due to dispersed redundant negative regulators. *Proc. Natl. Acad. Sci. USA* **102:**12147–12152.
83. **Kao, K. C., and G. Sherlock.** 2008. Molecular characterization of clonal interference during adaptive evolution in asexual populations of *Saccharomyces cerevisiae*. *Nat. Genet.* **40:**1499–1504.
84. **Kathe, S. D., R. Barrantes-Reynolds, P. Jaruga, M. R. Newton, C. J. Burrows, V. Bandaru, M. Dizdaroglu, J. P. Bond, and S. S. Wallace.** 2009. Plant and fungal Fpg homologs are formamidopyrimidine DNA glycosylases but not 8-oxoguanine DNA glycosylases. *DNA Repair* 8:643–653.
85. **Kolodner, R. D., and G. T. Marsischky.** 1999. Eukaryotic DNA mismatch repair. *Curr. Opin. Genet. Dev.* **9:** 89–96.
86. **Krogh, B. O., and L. S. Symington.** 2004. Recombination proteins in yeast. *Annu. Rev. Genet.* **38:**233–271.
87. **Larriba, G., and R. Calderone.** 2008. Heterozygosity and loss of heterozygosity in *Candida albicans*, p. 43–52. *In* G. San Blas and R. Calderone (ed.), *Pathogenic Fungi: Host Interactions and Emerging Strategies for Control*. Horizon Press, Wymondham, United Kingdom.
88. **Lasker, B. A., G. F. Carle, G. S. Kobayashi, and G. Medoff.** 1989. Comparison of the separation of *Candida albicans* chromosome-size DNA by pulse-field gel electrophoresis techniques. *Nucleic Acids Res.* **17:**3783–3793.
89. **Lawrence, C. W.** 2002. Cellular roles of DNA polymerase zeta and Rev1 protein. *DNA Repair* **1:**425–435.
90. **Legrand, M., C. L. Chan, P. A. Jauert, and D. T. Kirkpatrick.** 2008. Analysis of base excision and nucleotide excision repair in *Candida albicans*. *Microbiology* **154:** 2446–2456.
91. **Legrand, M., C. L. Chan, P. A. Jauert, and D. T. Kirkpatrick.** 2007. Role of DNA mismatch repair and double-strand break repair in genome stability and antifungal drug resistance in *Candida albicans*. *Eukaryot. Cell* **6:**2194–2205.

91a. **Legrand, M., C. L. Chan, P. A. Jauert, and D. T. Kirkpatrick.** 2011. The contribution of the S-phase checkpoint genes *MEC1* and SGS1 to genome stability maintenance in *Candida albicans*. *Fungal Genet. Biol.* **48:**823–830.

92. **Leng, P., P. E. Sudbery, and A. J. P. Brown.** 2000. Rad6 represses yeast-hypha morphogenesis in the human fungal pathogen *Candida albicans*. *Mol. Microbiol.* **35:**1264–1275.
93. **Lenormand, T., and S. P. Otto.** 2000. The evolution of recombination in a heterogeneous environment. *Genetics* **156:**423–438.
94. **Lephart, P. R., and P. T. Magee.** 2006. Effect of the major repeat sequence on mitotic recombination in *Candida albicans*. *Genetics* **174:**1737–1744.
95. **Lephart, P. R., H. Chibana, and P. T. Magee.** 2005. Effect of the major repeat sequence on chromosome loss in *Candida albicans*. *Eukaryot. Cell* **4:**733–741.
96. **Lin, C. Y., Y. C. Chen, H. J. Lo, K. W. Chen, and S. Y. Li.** 2007. Assessment of *Candida glabrata* strain relatedness by pulsed-field gel electrophoresis and multilocus sequence typing. *J. Clin. Microbiol.* **45:**2452–2459.
97. **Llorente, B., C. E. Smith, and L. S. Symington.** 2008. Break-induced replication: what is it and what is it for? *Cell Cycle* **7:**859–864.
98. **Lockhart, S. R., J. J. Fritch, A. S. Meier, K. Schroppel, T. Srikantha, R. Galask, and D. R. Soll.** 1995. Colonizing populations of *Candida albicans* are clonal in origin but undergo microevolution through C1 fragment reorganization as demonstrated by DNA fingerprinting and C1 sequencing. *J. Clin. Microbiol.* **33:**1501–1509.
99. **Lydeard, J. R., S. Jain, M. Yamaguchi, and J. E. Haber.** 2007. Break-induced replication and telomerase-independent telomere maintenance require Pol32. *Nature* **448:**820–823.
100. **Lynch, M., W. Sung, K. Morris, N. Coffey, C. R. Landry, E. B. Dopman, W. J. Dickinson, K. Okamoto, S. Kulkarni, D. L. Hartl, and W. K. Thomas.** 2008. A genome-wide view of the spectrum of spontaneous mutations in yeast. *Proc. Natl. Acad. Sci. USA* **105:**9272–9277.
101. **Lyndaker, A. M., and E. Alani.** 2010. A tale of tails: insights into the coordination of 3′ end processing during homologous recombination. *Bioessays* **31:**315–321.
102. **Mable, B. K., and S. P. Otto.** 2001. Masking and purging mutations following EMS treatment in haploid, diploid and tetraploid yeast (*Saccharomyces cerevisiae*). *Genet. Res.* **77:**9–26.
103. **Magee, B. B., M. D. Sanchez, D. Saunders, D. Harris, M. Berriman, and P. T. Magee.** 2008. Extensive chromosome rearrangements distinguish the karyotype of the hypovirulent species of *Candida dubliniensis* from the virulent *Candida albicans*. *Fungal Genet. Biol.* **45:**338–350.
104. **Magee, B. B., and P. T. Magee.** 1987. Electrophoretic karyotypes and chromosome numbers in *Candida species*. *J. Gen. Microbiol.* **133:**425–430.
105. **Magee, P. T.** 2007. Genomic structure and dynamics in *Candida albicans*, p. 7–28. *In* C. d'Enfert and B. Hube (ed.), Candida*: Comparative and Functional Genomics*. Caister Academic Press, Norfolk, United Kingdom.
106. **Malkova, A., E. L. Ivanov, and J. E. Haber.** 1996. Double-strand break repair in the absence of *RAD51* in yeast: a possible role for break-induced replication. *Proc. Natl. Acad. Sci. USA* **93:**7131–7136.
107. **Malkova, A., M. L. Naylor, M. Yamaguchi, G. Ira, and J. E. Haber.** 2005. *RAD51*-dependent break-induced replication differs in kinetics and checkpoint responses from *RAD51*-mediated gene conversion. *Mol. Cell. Biol.* **25:**933–944.
108. **Mandegar, M. A., and S. P. Otto.** 2007. Mitotic recombination counteracts the benefits of genetic segregation. *Proc. Biol. Sci.* **274:**1301–1307.

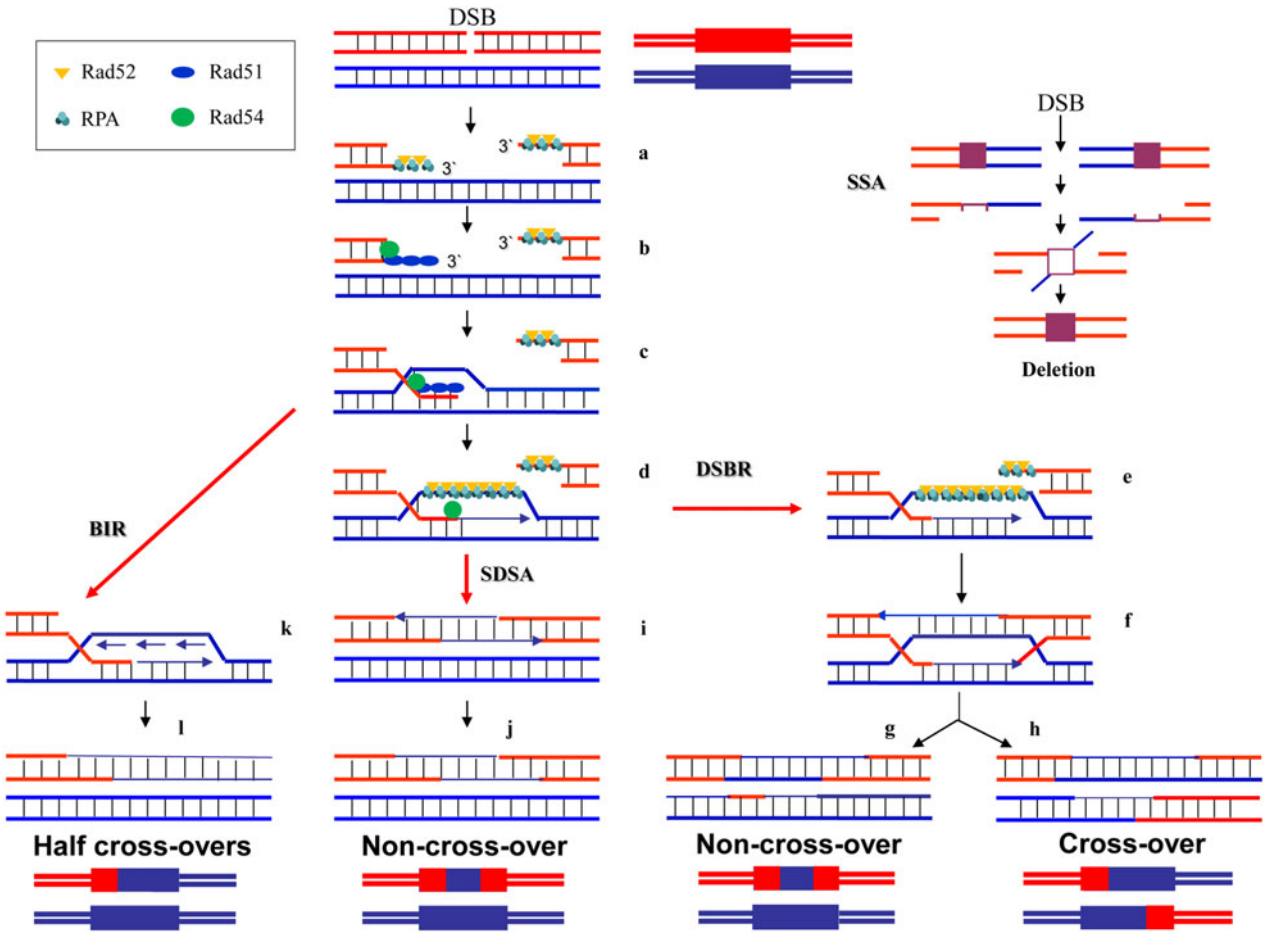
108a. **Mansfield, B. E., H. N. Oltean, B. G. Oliver, S. J. Hoot, S. E. Leyde, L. Hedstrom, and T. C. White.** 2010. Azole drugs are imported by facilitated diffusion in *Candida albicans* and other pathogenic fungi. *PLoS Pathog.* **6:**e1001126.

109. **Marr, K. A., C. N. Lyons, K. Ha, T. R. Rustad, and T. C. White.** 2001. Inducible azole resistance associated with a heterogeneous phenotype in *Candida albicans*. *Antimicrob. Agents Chemother.* **45:**52–59.
110. **Martinez, S. L., and R. D. Kolodner.** 2010. Functional analysis of human mismatch repair gene mutations identifies weak alleles and polymorphisms capable of polygenic interactions. *Proc. Natl. Acad. Sci. USA* **107:** 5070–5075.
111. **Masutani, C., M. Araki, A. Yamada, R. Kusumoto, T. Nogimori, T. Maekawa, S. Iwai, and F. Hanaoka.** 1999. Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity. *EMBO J.* **18:**3491–3501.
112. **Mazin, A. V., A. A. Alexeev, and S. C. Kowalczykowski.** 2003. A novel function of Rad54 protein. Stabilization of the Rad51 nucleoprotein filament. *J. Biol. Chem.* **278:**14029–14036.

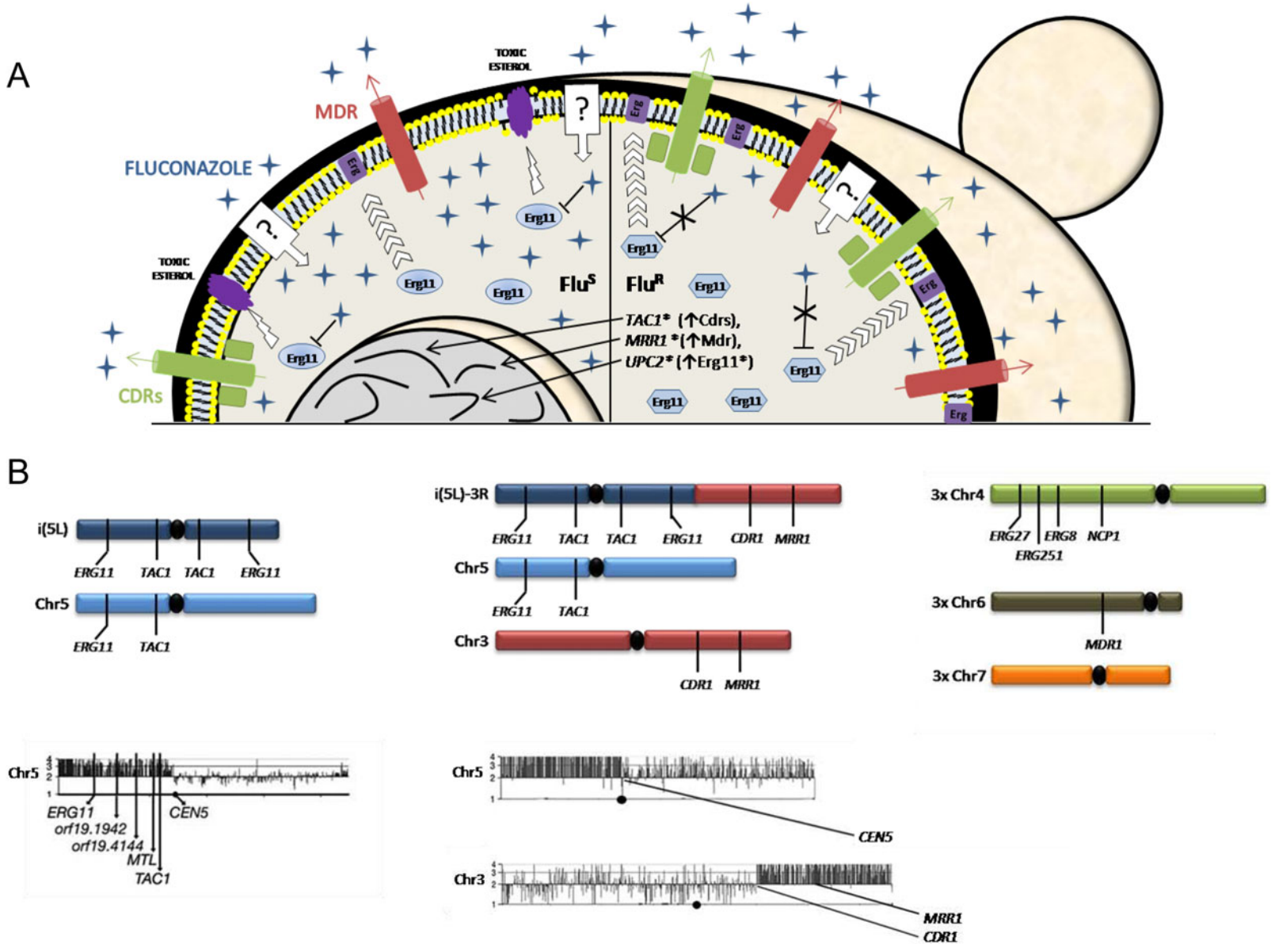
113. **McDonald, J. P., and R. Rothstein.** 1994. Unrepaired heteroduplex DNA in *Saccharomyces cerevisiae* is decreased in RAD1 Rad52 independent recombination. *Genetics* **137:**393–405.
114. **McEachern, M., and J. E. Haber.** 2006. Break-induced replication and recombinational telomere elongation in yeast. *Annu. Rev. Biochem.* **75:**111–135.
115. **Mimitou, E. P., and L. S. Symington.** 2009. DNA end resection: many nucleases make light work. *DNA Repair* **8:**983–995.
116. **Mishina, Y., E. M. Duguid, and C. He.** 2006. Direct reversal of DNA alkylation damage. *Chem. Rev.* **106:** 215–232.
117. **Modrich, P.** 2006. Mechanisms in eukaryotic mismatch repair. *J. Biol. Chem.* **281:**30305–30309.
118. **Morio, F., C. Loge, B. Besse, C. Hennequin, and P. Le Pape.** 2010. Screening for amino acid substitutions in the *Candida albicans* Erg11 protein of azole-susceptible and azole-resistant clinical isolates: new substitutions and a review of the literature. *Diagn. Microbiol. Infect. Dis.* **66:**373–384.
119. **Morrow, D. M., C. Connelly, and P. Hieter.** 1997. "Break copy" duplication: a model for chromosome fragment formation in *Saccharomyces cerevisiae*. *Genetics* **147:**371–382.
120. **Morschhauser, J.** 2010. Regulation of multidrug resistance in pathogenic fungi. *Fungal Genet. Biol.* **47:**94–106.
121. **Morschhauser, J., K. S. Barker, T. T. Liu, B. W. J. Bla, R. Homayouni, and P. D. Rogers.** 2007. The transcription factor Mrr1p controls expression of the *MDR1* efflux pump and mediates multidrug resistance in *Candida albicans*. *PLoS Pathog.* **3:**e164.
122. **Mott, C., and L. S. Symmington.** 2011. RAD51-independent inverted-repeat recombination by a strand-annealing mechanism. *DNA Repair* **10:**408–415.
123. **Navarro-Garcia, F., R. M. Perez-Diaz, B. B. Magee, J. Pla, C. Nombela, and P. Magee.** 1995. Chromosome reorganization in *Candida albicans* 1001 strain. *J. Med. Vet. Mycol.* **33:**361–366.
124. **Nelson, J. R., C. W. Lawrence, and D. C. Hinkle.** 1996. Deoxycytidyl transferase activity of yeast *REV1* protein. *Nature* **382:**729–731.
125. **Niimi, K., B. C. Monk, A. Hirai, K. Hatakenaka, T. Umeyama, E. Lamping, K. Maki, K. Tanabe, T. Kamimura, F. Ikeda, Y. Uehara, R. Kano, A. Hasegawa, R. D. Cannon, and M. Niimi.** 2010. Clinically significant micafungin resistance in *Candida albicans* involves modification of a glucan synthase catalytic subunit GSC1 (FKS1) allele followed by loss of heterozygosity. *J. Antimicrob. Chemother.* **65:**842–852.
126. **Nimonkar, A. V., R. A. Sica, and S. C. Kowalczykowski.** 2009. Rad52 promotes second-end DNA capture in double-stranded break repair to form complement-stabilized joint molecules. *Proc. Natl. Acad. Sci. USA* **106:**3077–3082.
127. **Noble, S., and A. D. Johnson.** 2007. Genetics of *Candida albicans*, a diploid human fungal pathogen. *Annu. Rev. Genet.* **14:**423–426.
128. **Odds, F. C.** 2010. Molecular phylogenetics and epidemiology of *Candida albicans*. *Future Microbiol.* **5:**67–79.
129. **Odds, F. C., M. E. Bougnoux, D. J. Shaw, J. M. Bain, A. D. Davidson, D. Diogo, M. D. Jacobsen, M. Lecomte, S. Y. Li, A. Tavanti, M. C. Maiden, N. A. Gow, and C. d'Enfert.** 2007. Molecular phylogenetics of *Candida albicans*. *Eukaryot. Cell* **6:**1041–1052.
130. **Odds, F. C., A. D. Davidson, M. D. Jacobsen, A. Tavanti, J. A. Whyte, C. C. Kibbler, D. H. Ellis, M. C. Maiden, D. J. Shaw, and N. A. Gow.** 2006. *Candida albicans* strain maintenance, replacement, and microvariation demonstrated by multilocus sequence typing. *J. Clin. Microbiol.* **44:**3647–3658.
131. **Oliver, B. G., J. L. Song, J. H. Choiniere, and T. C. White.** 2007. *cis*-Acting elements within the *Candida albicans ERG11* promoter mediate the azole response through transcription factor Upc2p. *Eukaryot. Cell* **6:**2231–2239.
132. **Otto, S. P., and A. C. Gerstein.** 2008. The evolution of haploidy and diploidy. *Curr. Biol.* **18:**R1121–R1124.
133. **Pannunzio, N. R., G. M. Manthey, and A. M. Bailis.** 2008. RAD59 is required for efficient repair of simultaneous double-strand breaks resulting in translocations in *Saccharomyces cerevisiae*. *DNA Repair* **7:**788–800.
134. **Pannunzio, N. R., G. M. Manthey, and A. M. Bailis.** 2010. *RAD59* and *RAD1* cooperate in translocation formation by single-strand annealing in *Saccharomyces cerevisiae*. *Curr. Genet.* **56:**87–100.
135. **Paques, F., and J. E. Haber.** 1999. Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **63:**349–404.
136. **Pardo, B., B. Gomez-Gonzalez, and A. Aguilera.** 2009. DNA repair in mammalian cells: DNA double-strand break repair: how to fix a broken relationship. *Cell Mol. Life Sci.* **66:**1039–1056.
137. **Perea, S., J. L. Lopez-Ribot, B. L. Wickes, W. R. Kirkpatrick, O. P. Dib, S. P. Bachmann, S. M. Keller, M. Martinez, and T. F. Patterson.** 2002. Molecular mechanisms of fluconazole resistance in *Candida dubliniensis* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrob. Agents Chemother.* **46:**1695–1703.
138. **Perepnikhatka, V., F. J. Fischer, M. Niimi, R. A. Baker, R. D. Cannon, Y. K. Wang, F. Sherman, and E. Rustchenko.** 1999. Specific chromosome alterations in fluconazole-resistant mutants of *Candida albicans*. *J. Bacteriol.* **181:**4041–4049.
139. **Perlin, D. S.** 2007. Resistance to echinocandin-class antifungal drugs. *Drug Resist. Update* **10:**121–130.
140. **Pohl, T. J., and J. A. Nickoloff.** 2008. Rad51-independent interchromosomal double-strand break repair by gene conversion requires Rad52 but not Rad55, Rad57, or Dmc1. *Mol. Cell. Biol.* **28:**897–906.
141. **Polakova, S., C. Blume, J. A. Zarate, M. Mentel, D. Jorck-Ramberg, J. Stenderup, and J. Piskur.** 2009. Formation of new chromosomes as a virulence mechanism in yeast *Candida glabrata*. *Proc. Natl. Acad. Sci. USA* **106:**2688–2693.
142. **Ponder, R. G., N. C. Fonville, and S. M. Rosenberg.** 2005. A switch from high-fidelity to error-prone DNA double-strand break repair underlies stress-induced mutation. *Mol. Cell* **19:**791–804.
143. **Prado, F., F. Cortes-Ledesma, P. Huertas, and A. Aguilera.** 2003. Mitotic recombination in *Saccharomyces cerevisiae*. *Curr. Genet.* **42:**185–198.
144. **Prakash, S., R. E. Johnson, and L. Prakash.** 2005. Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu. Rev. Biochem.* **74:**317–353.
145. **Prakash, S., and L. Prakash.** 2000. Nucleotide excision repair in yeast. *Mutat. Res.* **451:**13–24.
146. **Pujol, C., J. Reynes, F. Renaud, M. Raymond, M. Tibayrenc, F. J. Ayala, F. Janbon, M. Mallie, and J. M. Bastide.** 1993. The yeast *Candida albicans* has a clonal mode of reproduction in a population of infected human immunodeficiency virus-positive patients. *Proc. Natl. Acad. Sci. USA* **90:**9456–9459.
147. **Putnam, C. D., T. K. Hayes, and R. D. Kolodner.** 2009. Specific pathways prevent duplication-mediated genome rearrangements. *Nature* **460:**984–989.

148. **Rattray, A. J., and L. S. Symington.** 1994. Use of a chromosomal inverted repeat to demonstrate that the *RAD51* and *RAD52* genes of *Saccharomyces cerevisiae* have different roles in mitotic recombination. *Genetics* **138:**587–595.
149. **Rustad, T. R., D. A. Stevens, M. A. Pfaller, and T. C. White.** 2002. Homozygosity at the *Candida albicans MTL* locus associated with azole resistance. *Microbiology* **148:**1061–1072.
150. **Rustchenko, E.** 2007. Chromosome instability in *Candida albicans*. *FEMS Yeast Res*. **7:**2–11.
151. **Rustchenko, E. P., and F. Sherman.** 2003. Genetic instability of *Candida albicans*, p. 723–777. *In* D. H. Howard (ed.), *Pathogenic Fungi in Humans and Animals*, 2nd ed. Marcel Dekker Inc., New York, NY.
152. **Rustchenko, E. P., D. H. Howard, and F. Sherman.** 1997. Variation in assimilating functions occurs in spontaneous *Candida albicans* mutants having chromosomal alterations. *Microbiology* **143:**1765–1778.
153. **Rustchenko, E. P., D. H. Howard, and F. Sherman.** 1994. Chromosomal alterations of *Candida albicans* are associated with the gain and loss of assimilating functions. *J. Bacteriol.* **176:**3231–3241.
154. **Rustchenko-Bulgac, E. P., and D. H. Howard.** 1993. Multiple chromosomal and phenotypic changes in spontaneous mutants of *Candida albicans*. *J. Gen. Microbiol.* **139:**1195–1207.
155. **Rustchenko-Bulgac, E. P.** 1991. Variations of *Candida albicans* electrophoretic karyotypes. *J. Bacteriol.* **173:** 6586–6596.
156. **Rustchenko-Bulgac, E. P., F. Sherman, and J. B. Hicks.** 1990. Chromosomal rearrangements associated with morphological mutants provide a means for genetic variation of *Candida albicans*. *J. Bacteriol.* **172:**1276–1283.
157. **Sancar, G. B.** 1985. Expression of a *Saccharomyces cerevisiae* photolyase gene in *Escherichia coli*. *J. Bacteriol.* **161:**769–771.
158. **Sanglard, D.** 2007. Genomic view on antifungal resistance mechanisms among yeasts and fungal pathogens, p. 359–381. *In* C. d'Enfert and B. Hube (ed.), Candida: *Comparative and Functional Genomics*. Caister Academic Press, Norfolk, United Kingdom.
159. **Sanyal, K., M. Baum, and J. Carbon.** 2004. Centromeric DNA sequences in the pathogenic yeast *Candida albicans* are all different and unique. *Proc. Natl. Acad. Sci. USA* **101:**11374–11379.
160. **Scherer, S., and P. T. Magee.** 1990. Genetics of *Candida albicans*. *Microbiol. Rev.* **54:**226–241.
161. **Schoustra, S. E., A. J. Debets, M. Slakhorst, and R. F. Hoekstra.** 2007. Mitotic recombination accelerates adaptation in the fungus *Aspergillus nidulans*. *PLoS Genet*. **3:**e68.
162. **Schubert, S., P. D. Rogers, and J. Morschhauser.** 2008. Gain-of-function mutations in the transcription factor *MRR1* are responsible for overexpression of the *MDR1* efflux pump in fluconazole-resistant *Candida dubliniensis* strains. *Antimicrob. Agents Chemother*. **52:**4274–4280.
163. **Selmecki, A., A. Forche, and J. Berman.** 2010. Genomic plasticity of the human fungal pathogen *Candida albicans*. *Eukaryot. Cell* **9:**991–1008.
164. **Selmecki, A., M. Gerami-Neraj, C. Paulson, A. Forche, and J. Berman.** 2008. An isochromosome confers drug resistance *in vivo* by amplification of two genes, *ERG11* and *TAC1*. *Mol. Microbiol.* **68:**624–641.
165. **Selmecki, A., A. Forche, and J. Berman.** 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science* **313:**367–370.
166. **Selmecki, A., S. Bergmann, and J. Berman.** 2005. Comparative genome hybridization reveals widespread aneuploidy in *Candida albicans* laboratory strains. *Mol. Microbiol.* **55:**1553–1565.
167. **Selmecki, A. M., K. Dulmage, L. E. Cowen, J. B. Anderson, and J. Berman.** 2009. Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance. *PLoS Genet*. **5:**e1000705.
168. **Shin, J. H., M. J. Chae, J. W. Song, S. I. Jung, D. Cho, S. J. Kee, S. H. Kim, M. G. Shin, S. P. Suh, and D. W. Ryang.** 2007. Changes in karyotype and azole susceptibility of sequential bloodstream isolates from patients with *Candida glabrata* candidemia. *J. Clin. Microbiol.* **45:**2385–2391.
169. **Shinohara, A., H. Ogawa, and T. Ogawa.** 1992. Rad51 protein involved in repair and recombination in *S. cerevisiae* is a RecA-like protein. *Cell* **69:**457–470.
170. **Silver, P. M., B. G. Oliver, and T. C. White.** 2004. Role of *Candida albicans* transcription factor Upc2p in drug resistance and sterol metabolism. *Eukaryot. Cell* **3:**1391–1397.
171. **Smith, C. E., A. F. Lam, and L. S. Symington.** 2009. Aberrant double-strand break repair resulting in half crossvers in mutants defective in Rad51 or the DNA polymerase δ complex. *Mol. Cell. Biol.* **29:**1432–1441.
172. **Snell, R. G., I. F. Hermans, R. J. Wilkins, and B. E. Corner.** 1987. Chromosomal variations in *Candida albicans*. *Nucleic Acids Res*. **15:**3625.
173. **Solinger, J. A., K. Kiianitsa, and W. D. Heyer.** 2002. Rad54, a Swi2/Snf2-like recombinational repair protein, disassembles Rad51:dsDNA filaments. *Mol. Cell* **10:**1175–1188.
174. **Spampinato, C. P., R. L. Gomez, C. Galles, and L. D. Lario.** 2009. From bacteria to plants: a compendium of mismatch repair assays. *Mutat. Res*. **682:**110–128.
175. **Staib, P., M. Krestmar, T. Nichterlein, H. Hof, and J. Morschhäuser.** 2002. Host versus in vitro signals and intrastrain allelic differences in the expression of a *Candida albicans* virulence gene. *Mol. Microbiol.* **44:**1351–1366.
176. **Stellwagen, A. E., Z. W. Haimberger, J. R. Veatch, and D. E. Gottschling.** 2003. Ku interacts with telomerase RNA to promote telomere addition at native and broken chromosome ends. *Genes Dev.* **17:**2384–2395.
177. **Storchova, Z., A. Breneman, J. Cande, J. Dunn, K. Burbank, E. O'Toole, and D. Pellman.** 2006. Genome-wide analysis of polyploidy in yeast: scaling effects and genome stability. *Nature* **443:**541–547.
178. **Sugawara, N., and J. E. Haber.** 1992. Characterization of double-strand break-induced recombination: homology requirements and single-stranded DNA formation. *Mol. Cell. Biol.* **12:**563–575.
179. **Sugawara, N., G. Ira, and J. E. Haber.** 2000. DNA length dependence of the single-strand annealing pathway and the role of *Saccharomyces cerevisiae RAD59* in double-strand break repair. *Mol. Cell. Biol.* **20:**5300–5309.
180. **Sung, P.** 1997. Function of yeast Rad52 protein as a mediator between replication protein A and the Rad51 recombinase. *J. Biol. Chem.* **272:**28194–28197.
181. **Sung, P.** 1997. Yeast Rad55 and Rad57 proteins form a heterodimer that functions with replication protein A to promote DNA strand exchange by Rad51 recombinase. *Genes Dev.* **11:**1111–1121.
182. **Sung, P., and D. L. Robberson.** 1995. DNA strand exchange mediated by a RAD51-ssDNA nucleoprotein filament with polarity opposite to that of RecA. *Cell* **82:**453–461.
183. **Suzuki, T., I. Kobayashi, T. Kanbe, and K. Tanaka.** 1989. High frequency variation of colony morphology and chromosome reorganization in the pathogenic yeast *Candida albicans*. *J. Gen. Microbiol.* **135:**425–434.
184. **Symington, L. S.** 2002. Role of RAD52 epistasis group genes in homologous recombination and double-strand break repair. *Microbiol. Mol. Biol. Rev.* **66:**630–670.

185. **Tavanti, A., N. A. Gow, M. C. Maiden, F. C. Odds, and D. J. Shaw.** 2004. Genetic evidence for recombination in *Candida albicans* based on haplotype analysis. *Fungal Genet. Biol.* **41:**553–562.
186. **Torres, E. M., T. Sokolsky, C. M. Tucker, L. Y. Chan, M. Boselli, M. J. Dunham, and A. Amon.** 2007. Effects of aneuploidy on cellular physiology and cell division in haploid yeast. *Science* **317:**916–924.
187. **van het Hoog, M., T. J. Rast, M. Martchenko, S. Grindle, D. Dignard, H. Hogues, C. Cuomo, M. Berriman, S. Scherer, B. B. Magee, M. Whiteway, H. Chibana, A. Nantel, and P. T. Magee.** 2007. Assembly of the *Candida albicans* genome into sixteen supercontigs aligned on the eight chromosomes. *Genome Biol.* **8:**R52.
188. **Van Houten, B., D. L. Croteau, M. J. DellaVecchia, H. Wang, and C. Kisker.** 2005. "Close-fitting sleeves": DNA damage recognition by the UvrABC nuclease system. *Mutat. Res.* **577:**92–117.
189. **White, T. C.** 1997. The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14α demethylase in *Candida albicans*. *Antimicrob. Agents Chemother.* **41:** 1488–1494.
190. **Xiao, W., and L. Samson.** 1992. The *Saccharomyces cerevisiae MGT1* DNA repair methyltransferase gene: its promoter and entire coding sequence, regulation and in vivo biological functions. *Nucleic Acids Res.* **20:** 3599–3606.
191. **Yoshida, J., K. Umezu, and H. Maki.** 2003. Positive and negative roles of homologous recombination in the maintenance of genome stability in *Saccharomyces cerevisiae*. *Genetics* **164:**31–46.
192. **Zheng, X., Y. Wang, and Y. Wang.** 2004. Hgc1, a novel hypha-specific G1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. *EMBO J.* **23:** 1845–1856.



COLOR PLATE 1 (CHAPTER 5) Molecular models for DSBR, SDSA, BIR, and SSA pathways of HR. According to current models, HR events are initiated by the introduction of a DSB in a DNA molecule. The ends of a DSB are resected by nucleases (MRX and Sae2, followed by the exonuclease Exo1 and/or the helicase-topoisomerase complex Sgs1-Top3-Rmi1) that leave 3' ssDNA overhanging ends (115) which are first coated by RPA to prevent formation of DNA secondary structures (a). RPA recruits Rad52, which, in turn, displaces RPA, at the time that it interacts with Rad51 and facilitates the formation of a Rad51-ssDNA right-handed helical nucleoprotein filament (b). Other proteins, such as Rad55 and Rad57, may also help in the formation of the filament (180–182) (not shown). The Rad51 filament locates a homologous DNA donor and with the help of Rad54 causes chromatin remodeling, DNA unwinding, and strand exchange with the homologous partner (112) (c). This process generates a displaced strand, which is a substrate for RPA and Rad52, and forms a structure known a D loop (c). Then Rad51 is displaced through the action of Rad54, and the 3' end of the invading strand becomes a substrate for elongation by DNA polymerases (173) (d). Synthesis of DNA results in further DNA displacement and binding of RPA and Rad52. BIR may occur when only one end of the DSB has homology to the template or the other end is lost (left column); the homologous end can undergo strand invasion into a homologous or nonhomologous chromosome, forming a replication fork (k); long segments of the template that can extend until the telomere are then replicated, resulting in long tracts of GC (l). The final product contains the undamaged molecule and one of the two molecules resulting from a CO in DSBR (half-CO). When both ends of the DSB have homology to the template, two events can occur. In SDSA the newly synthesized band dissociates from the template and reanneals to the other resected DNA end (i). After DNA synthesis and ligation, the recombined molecules are resolved as non-COs (j). Alternatively, the displaced strand now captures the second resected end and anneals it to the D loop (second-end capture), a process promoted by Rad52 (126) (e). DNA synthesis from the second end and ligation result in a double Holliday junction (f) which is resolved as either non-COs (g) or COs (h). When a DSB occurs between direct repeats, recombination may take place by SSA (upper right). Resection of the 5' ends allows annealing of the direct repeats of both DNA molecules; this event is followed by resection of the 3' overhanging ends by the endonuclease Rad1-Rad10 (see text), DNA synthesis, and ligation. SSA results in the loss of one of the direct repeats and the intervening sequence. 10.1128/9781555817176.ch5cp1



COLOR PLATE 2 (CHAPTER 5) Mechanisms of fluconazole resistance in *Candida albicans* due to aneuploidies. (Top left) Fluconazole-susceptible (Flu^S) *C. albicans*. Fluconazole (blue star) enters the cell by facilitated diffusion (Mansfield et al., 10th Candida and Candidiasis, 2010, ASM Conferences, Miami, FL). Efflux pumps *CDR1* (green) and *MDR1* (red) excrete fluconazole out of the cell. Sensitive Erg11 (lanosterol demethylase) (blue ellipse) is inhibited by fluconazole, and this results in the synthesis of toxic sterols that destabilize the plasma membrane. (Top right) Flu^R *C. albicans* cell. The asterisk after the Tac1, Mrr1, and Erg11 indicates that the corresponding gene is in homozygosis. Mutated Erg11 that has become Flu^R is indicated as a hexagon. (Bottom) Schemes of isochromosome i(5L) and a chimerical chromosome (167). The presence of an extra i(5L) implies an increase in the copy number of both *ERG11* and transcriptional activator *TAC1*. The presence of an extra chimerical chromosome i(5L)-3R implies also an additional increase in the copy number of *MRR1*. Other genes related to fluconazole resistance are indicated in chromosomes 4, 6, and 7 (as described in reference 167). 10.1128/9781555817176.ch5cp2

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch6

6

Switching and Mating

DAVID R. SOLL

INTRODUCTION

Up until 1999, *Candida albicans* was assumed to be asexual, reproducing clonally. No one had identified sex genes, such as those in the related hemiascomycete *Saccharomyces cerevisiae*, and no one had described "shmooing," the formation of conjugation projections, or fusants, the fusion of cells of opposite mating type through these projections. Studies of the population structure of *C. albicans* had revealed primarily a picture of clonal propagation (27, 33, 72, 77, 80, 113), but there were some indications that this conclusion was not foolproof. Although most studies on population structure revealed that propagation was primarily clonal, low levels of recombination were suggested. Of course, without an identified genetic basis for mating, these rare recombinational events could be attributed to nonsexual fusions, mitotic recombination, or even horizontal gene transfer. However, one observation by Sadhu and coworkers (88) made many wonder if the mating system had been missed. They found that *CAG1*, a *C. albicans* ortholog to the gene encoding the alpha subunit of the *S. cerevisiae* trimeric G protein complex, Gbal, rescued the *S. cerevisiae* null mutant of Gbal. Although provocative, the trimeric G protein complex could have multiple functions, which could explain this early result without eliciting the possibility of a cryptic mating system.

But the question of a sexual cycle was erased in 1999 when the genes for a mating system harbored in a single mating-type locus, *MTL*, were identified from the sequencing data of *C. albicans* that were emerging from the *C. albicans* genome project centered at Stanford and the University of Minnesota (16, 44). In scrutinizing these data, Hull and Johnson (40) identified an **a** and α copy of this locus in what proved to be an **a**/α strain. Hull and Johnson (40) found orthologs of three *S. cerevisiae* mating-type genes in the *C. albicans MTL* locus. Although the scheme for mating and the regulation of the response to the mating pheromones proved in subsequent studies to be in general similar to that of *S. cerevisiae* and hence that of the hemiascomycetes in general (17), the biggest surprise was the fundamental and unique role that white-opaque switching (96) played in the mating process of *C. albicans*. Subsequently the same role for switching was demonstrated in the closely related species *Candida dubliniensis* (79). For that reason, mating in *C. albicans* cannot be discussed without giving significant attention to switching.

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THE MATING LOCUS OF *C. ALBICANS*

Hull and Johnson (40) found that the laboratory strain of *C. albicans*, SC5314, which was in the process of being sequenced was heterozygous at a mating-type locus that they called the "mating type-like" (*MTL*) locus, since its role in mating had not been demonstrated at the time and because of its similarity to the locus of *S. cerevisiae*. Because the *MTL* locus has proven to be a mating locus, it is now referred to simply as that. In an *MTL* heterozygous strain (**a**/α), there is one **a** and one α copy of the locus, *MTL***a** and *MTL*α (Fig. 1A), one on each of the chromosome 5 homologs (93, 120). The *MTL***a** locus contains two mating genes, *MTL***a***1* and *MTL***a***2* (114), and the *MTL*α locus contains two mating genes, *MTL*α*1* and *MTL*α*2* (Fig. 1A). Mtl**a**1 (**a**1) and Mtlα2 (α2) combine to form a corepressor complex, **a**1-α2, which suppresses **a** and α gene expression, and mating, in **a**/α cells (Fig. 1D), as it does in *S. cerevisiae* (25, 69). To become mating competent, *C. albicans* **a**/α cells, which represent a majority of strains in general (53, 72, 86, 113), must undergo homozygosis to **a**/**a** or α/α (41, 54, 58). The gene compositions of the *MTL* locus of **a**/**a** and α/α cells are presented in Fig. 1B and C, respectively. Homozygosis leads to the loss of **a**1/α2 repression. Mtl**a**2 (**a**2) induces **a** genes and mating in **a**/**a** cells (Fig. 1E), and Mtlα1 (α1) induces α genes and mating in α/α cells (Fig. 1F) (114). While *C. albicans* **a**1, α2, and α1 are homologous to their orthologs in *S. cerevisiae*, *C. albicans* **a**2 has no orthologs in *S. cerevisiae* (40, 114). In *S. cerevisiae* **a** cells, **a** genes are expressed by default, whereas in *C. albicans* **a**/**a** cells, **a**2 is an active inducer of expression. Based on a detailed comparative genomic analysis of the mating genes of the hemiascomycetes, it seems likely that the common ancestor of the hemiascomycetes, which includes the *Candida* group and the *Saccharomyces* group, contained not only the common **a**1, α2, and α1 genes but also the *C. albicans* **a**2 gene (17, 102). Hence, *S. cerevisiae* lost **a**2 activation of **a** genes and mating in **a**

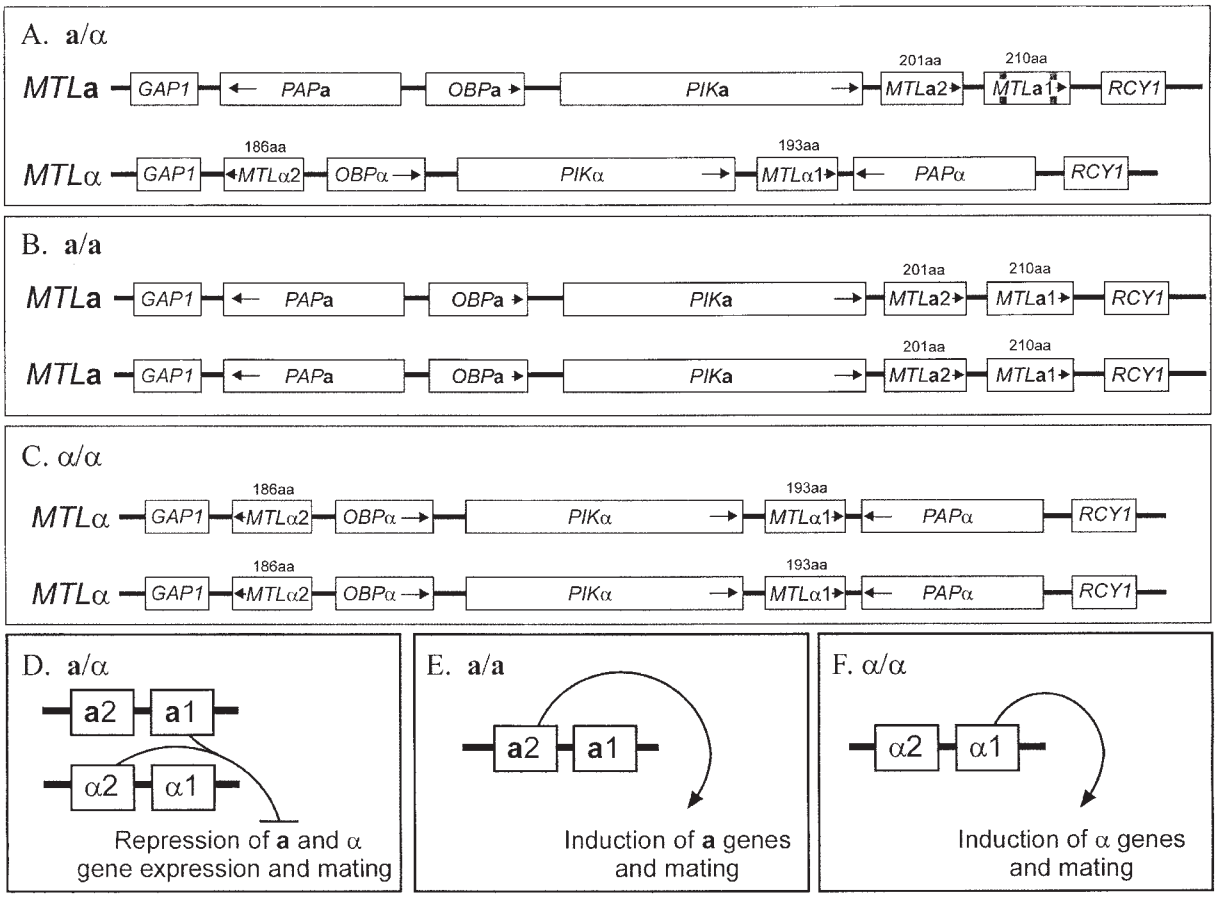

FIGURE 1 Genotype at the mating-type locus and basic regulation of mating types. (A) Mating-type locus of an **a**/α cell; (B) mating-type locus of an **a**/**a** cell; (C) mating-type locus of an α/α cell; (D) inhibition of a and α gene expression and mating by the **a**1-α2 complex in **a**/α cells; (E) induction of **a** genes and mating in **a**/**a** cells; (F) induction of α genes and mating in α/α cells. The genes in all panels are described in the text. 10.1128/9781555817176.ch6f1

cells and acquired a default mechanism for **a** gene expression.

And just as interesting, the C. *albicans* mating-type idiomorphs *MTL***a** and *MTL*α contain three additional genes: *PAP***a** and *PAP*α, respectively, which code for poly(A) polymerases; *PIK***a** and *PIK*α, respectively, which code for phosphatidylinositol kinases; and *OBP***a** and *OBP*α, respectively, which code for oxysterol-binding protein-like proteins (Fig. 1A) (40). What is interesting about these genes is, first, that they are embedded in the mating locus; second, that they do not match up along the *MTL***a** and *MTL*α homologs of chromosome 5; third, that they actually are not even in the same order; and fourth, that the **a** and α "alleles" are highly divergent compared to the other genes on chromosome 5 outside the *MTL* locus (C. Pujol, T. Srikantha, and D. R. Soll, unpublished observations). It is possible that they may have evolved distinct functions, which should caution C. *albicans* scientists from claiming that strains that are homozygous for the **a** locus are isogenic with strains that are homozygous for the α locus.

THE DEMONSTRATION OF MATING

One year after the identification of the mating locus, two different studies demonstrated mating at the genetic level. Hull et al. (41) used **a** and α strains, each carrying a different auxotrophic marker, *ade*$^-$ and *ura*$^-$, respectively, to demonstrate mating through complementation. Hull et al. (41) generated *MTL*-homozygous strains by deleting either *MTL* genes or the entire *MTL* locus. They performed their crosses in the mouse model for systemic infection. Magee and Magee (58) similarly used a complementation approach to demonstrate mating in vitro by inducing the loss of an entire chromosome 5 homolog by growing cells on sorbose as a sole carbon source.

Visualization of the actual fusion event at the cellular level, however, occurred only after it was discovered that opaque cells were of the mating-competent phenotype (54, 62). Using opaque rather than white cells provided mating frequencies several orders of magnitude higher than in the original studies (41, 58), which employed mating-incompetent white cells. Lockhart et al. (54) demonstrated by differential interference contrast microscopy, three-dimensional dynamic image analysis system reconstruction (99), vital staining with fluorescently tagged lectins, and direct fluorescent staining of septae and nuclei that in suspension cultures containing mixtures of **a**/**a** and α/α cells, evaginations were formed without constrictions at the mother cell junctions (Fig. 2A and B). The evaginations (shmoos) grew into conjugation tubes that fused end to end to form a zy-

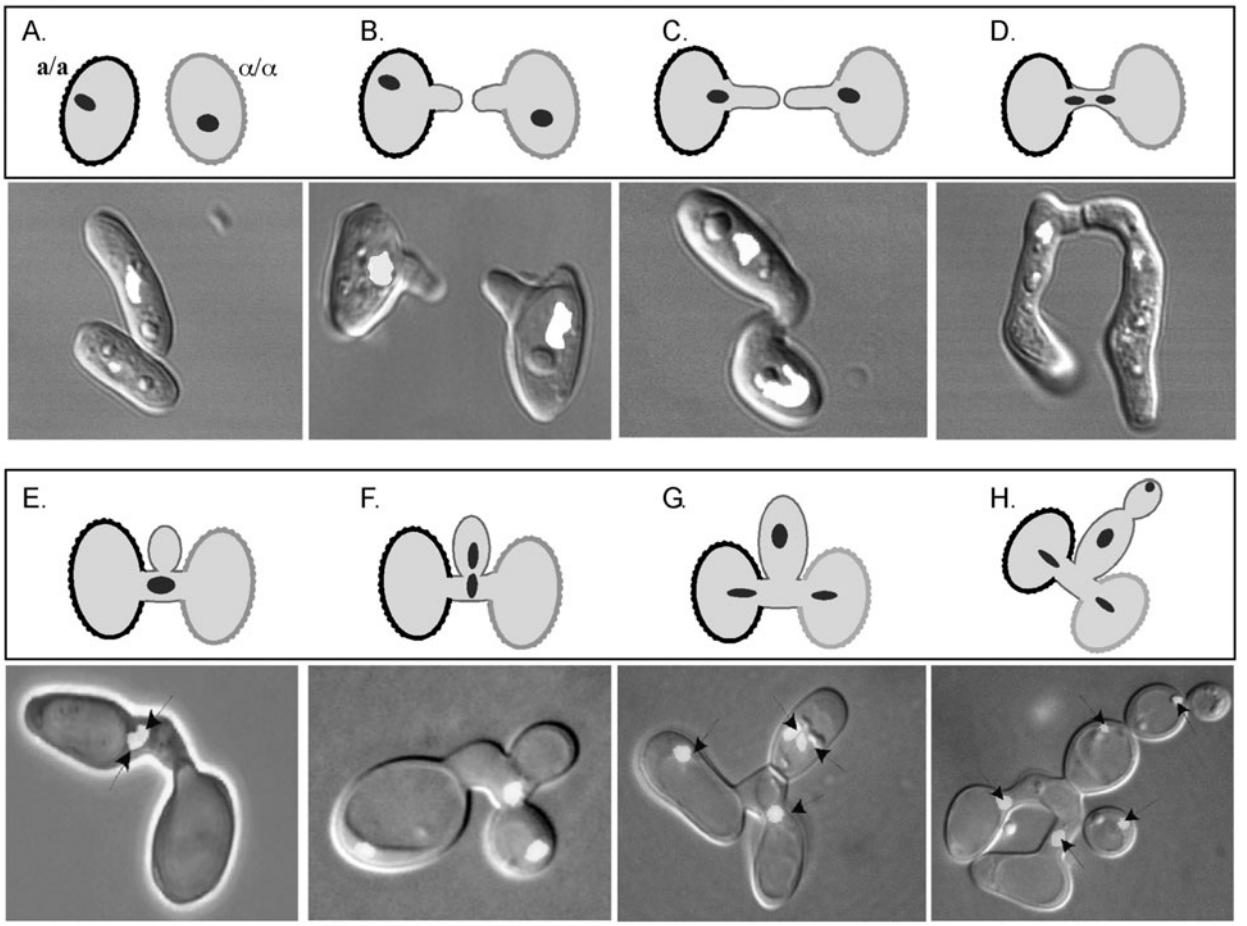

FIGURE 2 Cell biology of shmoo formation and fusion. (A) Initial opaque **a/a** and α/α cells; (B) shmoo formation, in which mating projections form; (C) chemotropism of tubes towards each other; (D) fusion of tubes; (E) fusion of daughter cell nuclei; (F) growth of daughter cell and division of tetraploid nucleus; (G) further nuclear chiasms; (H) budding of daughter cell. Arrows point to nuclei. 10.1128/9781555817176.ch6f2

gote connected by a conjugation bridge (Fig. 2C and D). From the conjugation tube, a daughter cell formed, which eventually separated through development of a septum (Fig. 2D and E). The nuclei of **a/a** and α/α cells moved into the conjugation tube and fused (9) (Fig. 2D and E). This tetraploid nucleus then divided by mitosis, with one daughter nucleus entering the daughter cell and the other remaining in the bridge (Fig. 2F). Subsequently, both nuclei divide and segregants are apparent in mating partners (Fig. 2G and H). Bennett et al. (9) demonstrated nuclear fusion by fluorescence-activated cell sorting and direct fluorescent staining. A similar scheme has been demonstrated for mating between the closely related species *Candida dubliniensis* (79). The mating process depicted in Fig. 3 is highly similar to that of *S. cerevisiae*, except that the final daughter cell is tetraploid rather than diploid. The entire process of attaining mating competence and mating is outlined in Fig. 3.

But tetraploidy seems to present a problem to *C. albicans*, given that meiosis has not been observed in this species. Bennett and Johnson (10) demonstrated that under nutrient-limiting conditions created either by incubating cells in minimal medium or by treating them with sorbose, both of which represent stress conditions, tetraploid cells randomly lost chromosomes. Some reverted to the diploid or near-diploid state. Using single nucleotide polymorphism and comparative genome hybridization techniques, Forche et al. (28) showed that as the tetraploids decrease ploidy, they can undergo chromosome shuffling and recombination. They further demonstrated that *SPO11*, which is necessary for recombination in *S. cerevisiae*, is required for recombination in *C. albicans* tetraploids. These authors note that a parasexual cycle involving chromosome loss rather than meiosis bypasses sporulation and that sporulation, which may have been lost, could be adverse to the survival of *C. albicans* as a commensal (28).

THE DISCOVERY OF THE SWITCHING REQUIREMENT

In 2002, Miller and Johnson (62) described a set of observations that connected white-opaque switching and mating in *C. albicans*. This added a new and unique aspect to *C. albicans* that truly distinguished it from mating in *S. cerevisiae* and, probably, from *Candida tropicalis* and other species in the CTG clade of the hemiascomycetes. Miller and Johnson (62) noted that aged colonies of **a/a** and α/α derivatives of laboratory strain CAI4 underwent white-opaque switching, but the parent **a/α** strain did not. They demonstrated that

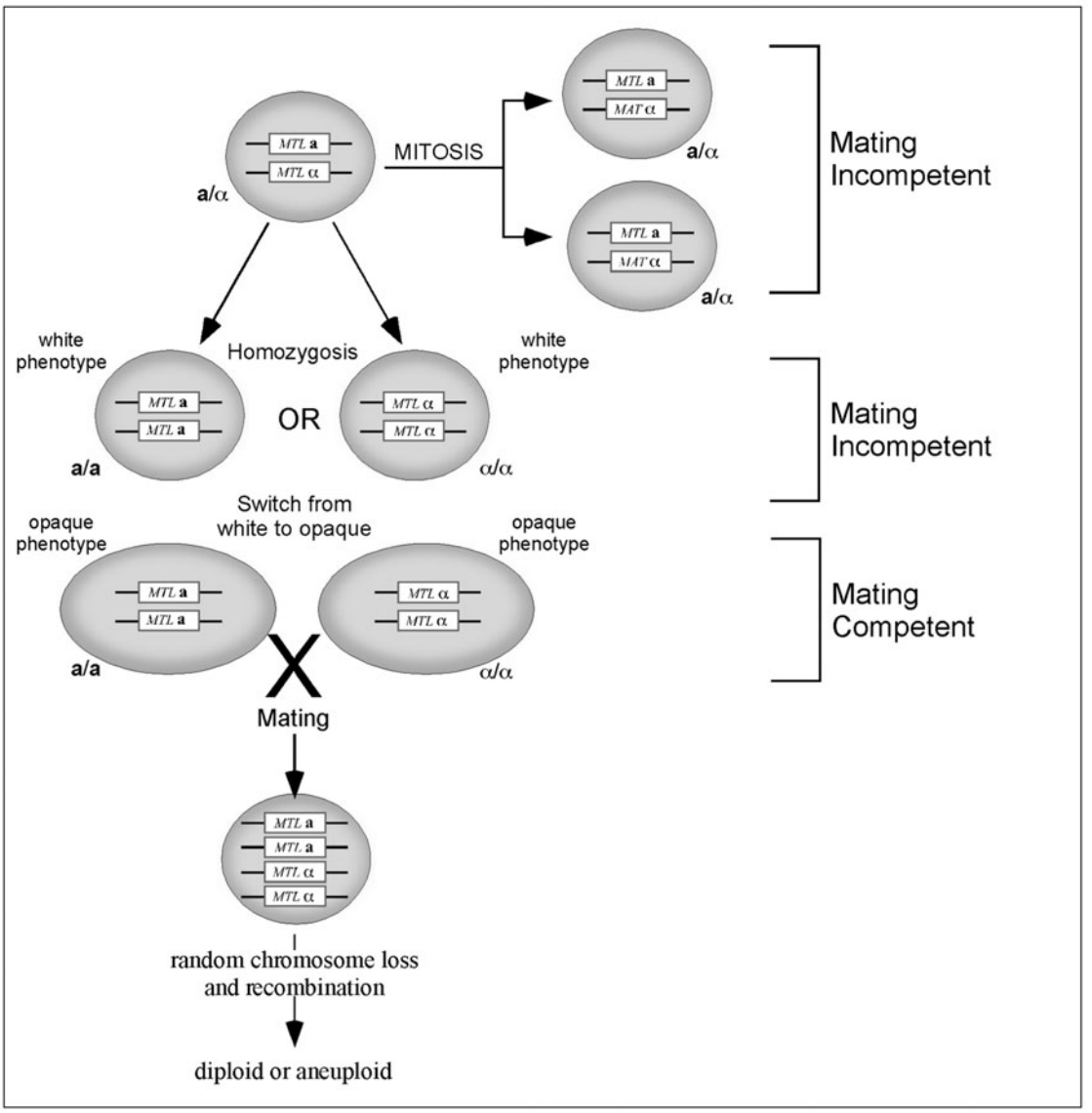

FIGURE 3 The mating process of C. *albicans* involves homozygosis at the *MTL* locus, a switch from white to opaque, mating, and the return to a diploid from a tetraploid state. Note that mating competency is attained through a switch from white to opaque. 10.1128/9781555817176.ch6f3

deletion of either *MTL***a**1 or *MTL*α2 resulted in switching, revealing that the same **a**1-α2 corepressor that repressed mating also repressed switching. Lockhart et al. (53) then showed that this held true for natural strains. Clinical **a**/α isolates did not undergo white-opaque switching unless they underwent *MTL* homozygosis, and clinical **a**/**a** and α/α isolates underwent switching.

In addition to the preceding discovery, Miller and Johnson (62) found in the same study that mixtures of opaque **a**/**a** and opaque α/α cells cross-streaked on agar underwent mating at frequencies many orders of magnitude higher than mixtures of white **a**/**a** and white α/α cells, or opaque **a**/**a** and white α/α cells, using the laboratory strain CAI4. This result indicated that mating depended upon switching and that the opaque phenotype, therefore, was the mating-competent phenotype. These results were verified and generalized by Lockhart et al. (54), who demonstrated that only the opaque cell phenotype of natural clinical strains was capable of mating. Lockhart et al. (54) provided microscopic evidence for crosses performed in suspension cultures. Again, it should be emphasized that since C. *albicans* and C. *dubliniensis* are the only hemiascomycetes that undergo white and opaque switching, the switching requirement for mating must have evolved in the ancestor of these two species some 20 million years ago (63). I return to this unique dependency and the reasons for it later in this chapter.

THE PHEROMONE RESPONSE

In 2003, three groups simultaneously demonstrated that chemically synthesized α-pheromone induced the mating response in **a**/**a** cells (8, 55, 74). The gene for α-pheromone, *MF*α*1* (74), could generate two possible pheromones, one 13 amino acids long and the other 14 amino acids long.

Both peptides proved functional, but the 13-mer proved more potent than the 14-mer. Unfortunately, as is the case for *S. cerevisiae* (19), the **a**-pheromone is highly modified, making it extremely difficult to chemically synthesize and to maintain. Additionally, the **a**-pheromone is highly adhesive to glass and plastic. Hence, Lockhart et al. (54) demonstrated the existence and function of **a**-pheromone by demonstrating that in a mixture of minority opaque **a/a** cells and majority opaque α/α cells, the latter cells shmoo presumably in response to **a**-pheromone produced by the minority **a/a** cells. Yi et al. (121) had even better results in a transmembrane experiment. The gene encoding the **a**-pheromone was recently identified (*MFA1*) by Dignard et al. (24).

The response of an opaque cell to pheromone produced by the opposite mating type was shown to be highly similar to that of *S. cerevisiae*, including the components of the major regulatory pathway that transduces the pheromone signal. The homologies include the receptors, components of the trimeric G protein complex, components of the mitogen-activated protein (MAP) kinase cascade, and the targeted transcription factor (Fig. 4). In the majority, but not in all cases, the orthologs are upregulated by pheromone in a similar fashion (8, 22, 55, 123). Where tested, these regulatory components in the transduction pathway play similar roles in the response of opaque cells to pheromone (18, 58, 121) (Fig. 4). The conservation of this entire pathway, from signal and receptor to target transcription factor, in the mating systems of the hemiascomycetes is quite remarkable, suggesting strong selective pressure. As is the case for *S. cerevisiae*, the pheromone response pathway genes *STE2*, *STE3*, *CAG1* (*S.c.GPA1*), and *CEK2* (*S.c.FUS3*) and the transcription factor *CPH1* (*S.c.STE12*) were upregulated by pheromone, but the pathway genes *STE4* and *CEK1* (*S.c.KSS1*) were selectively upregulated by pheromone in *C. albicans* but not *S. cerevisiae* (8, 123). On the other hand, *FAR1*, which plays a role in G_1 arrest, polarization, and gene expression, is constitutively expressed in *C. albicans* (121, 123) but upregulated by pheromone in *S. cerevisiae* (85). For the genes encoding the proteins involved in reentry into the mitotic cell cycle, karyogamy, pheromone adaptation, pheromone maturation, and pheromone transport, the majority are upregulated by pheromone in *C. albicans* as in *S. cerevisiae*, with two exceptions (*ECE1* and *RAM1*), which are upregulated in *C. albicans* but not in *S. cerevisiae* (123). On the whole, however, the repertoires of genes that are upregulated by pheromone in mating-competent **a** or α cells of *S. cerevisiae* and in mating-competent **a/a** or α/α cells of *C. albicans* are quite similar between the two species, as are their functions, suggesting a high degree of conservation.

Pheromone activates not only the MAP kinase pathway but also the factor arrest 1 gene, *FAR1*, which plays a role in cell polarity, G_1 arrest, and gene expression. In *S. cerevisiae*,

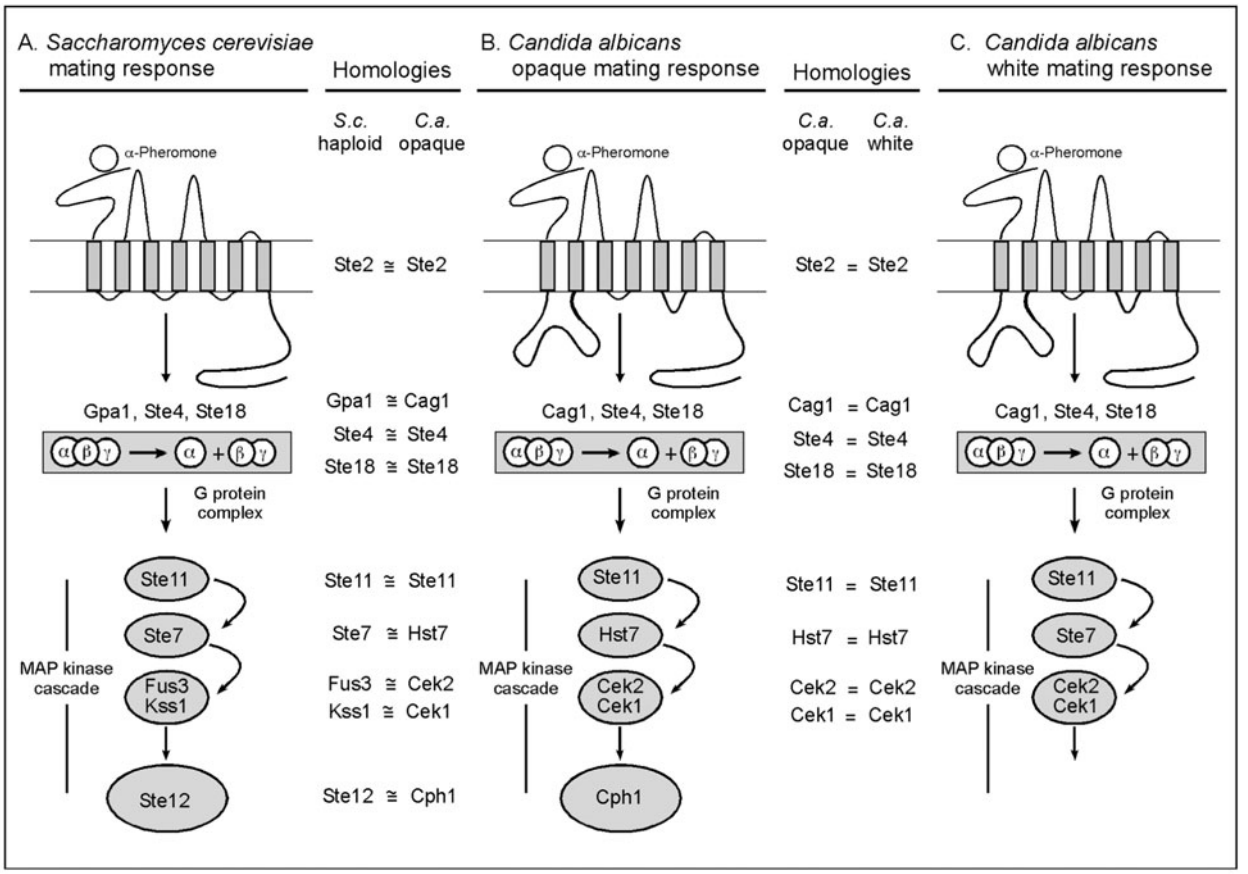

FIGURE 4 The pheromone response of *C. albicans* white cells shares the upper components of the conserved pheromone response pathways of *C. albicans* opaque cells and *S. cerevisiae* haploid cells in the mating process. Homology between components is noted by the symbol "=" and lack of homology by the symbol "≅". 10.1128/9781555817176.ch6f4

FAR1 is upregulated by pheromone, and the protein far1 is then activated by Fus3-dependent phosphorylation (20, 75, 83). Phosphorylated Far1 targets a protein complex containing Cdc24, Cdc42, and Bem1 to the membrane-localized trimeric G protein complex. In *C. albicans*, however, *FAR1* is expressed constitutively (123). As in *S. cerevisiae*, deletion of *FAR1* in *C. albicans* results in a defect in G_1 arrest that is readily demonstrated by fluorescent activated cell sorting and by nuclear staining techniques (121). This result is not obtained with the less precise halo assay (21), unless examined in the deletion mutant for *SST2*, which encodes a GTPase-activating protein that plays a role in desensitization to pheromone (23). Mutant *far1* cells exhibited a moderate decrease in the frequency of shmoo formation (121) and a dramatic decrease in the frequency of mating (21, 121). Therefore, Far1 is not absolutely essential for shmoo formation but is for the efficiency of the process. It does seem, however, to be almost, but not quite, essential for mating, as it is in *S. cerevisiae*. In *S. cerevisiae*, Roberts et al. (85) demonstrated that Far1 is essential for the expression of select genes in the pheromone response. Some, like *STE2*, are still upregulated by pheromone in the *far1* null mutant of *S. cerevisiae*, while others, like *STE4*, are not. Cote and Whiteway (21) found that all of the markers they examined by an oligomer microarray chip analysis were not upregulated in the double mutant *far1/far1 sst2/sst2*. Unfortunately, none of the genes that differed from *S. cerevisiae* expression were verified by an alternative method such as Northern hybridization, and furthermore, using an *sst2/sst2* background for the *far1/far1* mutation is problematic. Deletion of *SST2* in *S. cerevisiae* leads to a strong induction of *BAR1*, which degrades α-pheromone, leading to effects opposite those of the pheromone response (85). Hence, the *sst2* background may mask regulation of gene expression in response to pheromone in a *far1/far1* stain. Furthermore, gene expression in general has been shown to be affected in the *sst2* mutant of *S. cerevisiae* (85). Therefore, the role of Far1 in the regulation of gene expression by pheromone in opaque cells during the mating response still remains to be sorted out.

By analyzing a *FAR1* deletion mutant in a wild-type background of a natural strain, Yi et al. (121) showed that Far1 played no role in the white cell pheromone response or in the pheromone activation of white-specific genes, including selected ones of the pheromone response pathway. Moreover, in revisiting the role of Far1 in opaque cell gene regulation, Yi et al. (S. Yi, N. Sahni, and D. R. Soll, unpublished data) found that Cote and Whiteway (21) were not correct in their conclusion that Far1 was essential for pheromone induction of all mating-related genes, leading to a suggestion by the latter authors that there was recurrence of the circuitry and that the role of Far1 to some degree differed in *S. cerevisiae* and *C. albicans*. Roberts et al. (85) showed in supplemental data of *FAR1* function that in *S. cerevisiae*, deletion of *FAR1* abolished pheromone induction of just a subset of mating-related genes but had no effect on the induction of others. Yi et al. (unpublished) found that the role of Far1 in pheromone-induced expression of mating-related genes in opaque *C. albicans* cells was in fact highly similar to that in *S. cerevisiae*—i.e., it was necessary for some, but not all, of the pheromone-upregulated genes, the same result obtained in *S. cerevisiae* by Roberts et al. (85). Moreover, Yi et al. (unpublished) demonstrated that as is the case in *S. cerevisiae* (29), Far1, which is constitutively expressed in white and opaque cells, is modified post-transcriptionally in opaque cells, where it plays a major role in the pheromone response, and not in white cells, where it plays no role.

A set of genes involved in filamentation are also upregulated by pheromone in *C. albicans* opaque cells but not in haploid *S. cerevisiae* cells. The genes include some that are also employed in the pheromone response, like *CEK1*, *RBT1*, and *CPH1*, but also a number of genes like *FGR23*, *HWP1*, *SAP4*, *RBT4*, *SAP6*, *SAP5*, *ECE1*, *CZF1*, and *RIM101*, many of which do not have orthologs in *S. cerevisiae* (123). It has been suggested that these genes are involved in the formation of very long conjugation tubes formed by *C. albicans* in response to pheromone, which contrasts with the short projections formed by haploid *S. cerevisiae* cells in response to pheromone (123).

Finally, pheromone downregulates opaque-specific genes, most notably *OP4*, *SAP1*, and *SAP3* (55), even though the cell must first express the opaque phenotype to achieve mating competency. Pheromone does not upregulate white-specific genes (123). Hence, although mating requires a switch from white to opaque, it would appear that the cell must then suppress expression of some opaque genes to mate.

SAME-SEX MATING

C. albicans contains all of the functional components for mating between **a** and α strains that are conserved in diverse species in the hemiascomycetes (17, 50). Alby and coworkers (1) demonstrated by complementation that if the gene *BAR1*, which degrades the α-pheromone in **a/a** cells, is deleted, the mutant **a/a** cells can mate with each other at very low frequency. This "same-sex" mating was shown to require *Ste2*, the gene that encodes the receptor for the α-pheromone, and Mfα1, the gene that encodes the α-pheromone (1). Same-sex mating of **a/a** cells is not dependent on the **a** pheromone. The requirement for a *bar1/bar1* background, however, suggests that this may be a very rare event, since, as Heitman (34) points out, there have been no reports of natural *bar1/bar1* strains. Experiments in which auxotrophic **a/a** cells, control **a/a** cells, and α/α cells were mixed demonstrated that control cells can also mate at very low frequencies. This possible role of same-sex mating is extremely interesting and deserves further investigation.

SWITCHING PRIOR TO THE DISCOVERY OF ITS ROLE IN MATING

The spontaneous transition between the white and opaque phenotypes by a selected group of strains had been shown to affect a number of phenotypic and virulence traits before the discovery by Miller and Johnson (62) that it was an essential step in the mating process. In 1987, Slutsky et al. (96) identified white-opaque switching for the first time in a blood isolate, WO-1, that killed its host. In that original communication, it was demonstrated that opaque cells formed larger, flatter, smoother colonies than white cells; that opaque cells were sensitive to high and low temperature; that opaque cells were larger and longer than white cells; and that opaque cells were not induced to form hyphae by a shift to high pH, as were white cells (96). Within the next 5 years, studies revealed different rates of switching from white to opaque and opaque to white (12, 84), differences in adhesion and hydrophobicity (45, 116), inducibility by UV irradiation in both the white-to-opaque and opaque-to-white directions (66), a possible intermediate

phenotype in the cellular transition from white to opaque (12), differences in the morphology of the cell wall and the presence of a large opaque vacuole (4), the presence of hyphal and opaque-specific antigens on the wall of opaque cells (3), and selective stimulation of opaque cell filamentation by epithelial cells (2). Five years after the discovery of white-opaque switching, the first gene regulated by switching was identified, *PEP1* (67), an excreted aspartyl proteinase gene later found to be a member of a gene family and renamed *SAP1* for secreted aspartyl proteinase 1 (39, 118). Then, a second opaque-specific gene, *OP4* (68), and the first white-specific gene, *WH11* (104), were identified.

Since the early 1990s, a growing list of phenotypic characteristics, virulence traits, and expressed genes have been found to differ between white and opaque cells, the subject of several reviews that focus specifically on the switching process (56, 98, 100, 101). Some of the highlights are noted. First, it was demonstrated that whereas white-phase cells are more virulent in the mouse tail injection model for systemic infections (48), opaque cells were more proficient at colonizing the skin of newborn mice (47). Hence, the two phenotypes differed significantly in at least one aspect of virulence. Second, white cells could form thick biofilms on an elastomer surface, but opaque cells could not (22). Third, Geiger et al. (30) demonstrated that white cells released a potent chemoattractant that attracted human polymorphonuclear leukocytes, but opaque cells did not. Opaque cells appeared to be invisible to human white cells. And finally, Lan et al. (50) demonstrated by expression arrays that over 300 genes were upregulated or downregulated by the white-opaque transition. Thus, prior to the discovery by Miller and Johnson (62) of its role in mating, the white-opaque transition still remained a well-studied enigma, and for several reasons. First, it was not clear why opaque cells were unstable at physiological temperature (84, 96, 104). If the main niche of *C. albicans* is the vertebrate host (71), how could switching play a role, unless, as indicated by Kvaal et al. (47), switching was restricted to skin, the temperature of which is 32°C and thus supports the opaque phenotype? Second, estimates made in the late 1980s of the proportion of strains that underwent white-opaque switching were below 10% (D. R. Soll, unpublished observation), but in a study of vaginitis isolates, approximately one-fourth of the strains from different patients exhibited white-opaque switching (97).

MTL HOMOZYGOSIS AND THE MAINTENANCE OF OPAQUE CELLS IN THE HOST

As noted, the results of studies on *MTL* zygosity suggested that in nature the majority of strains were **a**/α (51, 53, 113). Interestingly, Odds et al. (72) found that *MTL*-homozygous strains were on average more resistant to azoles than **a**/α cells. Two other reports (31, 87) showed that fluconazole resistance was associated with *MTL* homozygosity. If *MTL*-homozygous strains were more drug resistant and some strains produced *MTL*-homozygous strains at high frequency (53), why didn't *MTL*-homozygous strains accumulate and predominate in nature? The answer may lie in the predominant mechanism leading to *MTL* homozygosis. Wu et al. (119) demonstrated that at least in vitro, the major mechanism for *MTL* homozygosis was the loss of one chromosome 5 homolog followed by duplication of the retained homolog. Mitotic recombination proved to be a second mechanism but far less frequent in vitro (119). Wu et al. (120) subsequently presented evidence suggesting that at least in the mouse tail injection model for systemic infection, **a**/α strains were on average more virulent than **a**/**a** or α/α strains. These observations led to the interesting hypothesis that if *MTL*-homozygous offspring do not mate after they are generated, they may disappear from the population, diluted out by the more competitive *MTL*-heterozygous strains. Moreover, tetraploids generated by mating have been shown to have reduced virulence in the mouse tail injection model (42), suggesting that if tetraploids did not reduce chromosome number, they too would be deleted from the population.

So how are minority **a**/**a** and α/α strains maintained in nature? This question is especially relevant when one considers that the production of these strains through *MTL* homozygosity is necessary for mating. Could it be that they prevail in locations where they have an advantage, such as when expressing the opaque phenotype on skin (47), or could it be that there are conditions in human niches that stabilize the mating-competent opaque phenotype?

SWITCHING, MATING, AND HOST ENVIRONMENTS

The sensitivity of the opaque phenotype to high temperature caused a conundrum regarding the relationship of host and mating. If *C. albicans* resides as a commensal or pathogen primarily in a vertebrate host, and the temperature for the majority of *C. albicans* niches is 37°C, which does not support the opaque phenotype, where does mating occur? Lachke et al. (49) appeared to provide a solution by showing that skin temperature is 32°C, at which opaque cell colonization and mating were facilitated. Indeed, in some areas of the skin of newborn mice upon which **a**/**a** and α/α cells were incubated, over 50% of all cells mated. However, these fusants did not produce daughter cells. And as previously asked, why would mating be restricted to skin, a minor host niche? The answer appeared to reside elsewhere. Characteristics of host tissue and the gastrointestinal tract were recently discovered to stabilize the opaque phenotype and even induce the white-to-opaque transition. It was initially suggested that the depletion of oxygen (anoxia) induced the white-to-opaque transition (26, 81), but subsequent experiments suggested that, in fact, it was increased CO_2, not O_2 depletion, that was responsible (37). In addition, it was demonstrated that *N*-acetylglucosamine (GlcNAc), found in the gastrointestinal tract, also induced white-to-opaque switching and stabilized the opaque phenotype (38). These discoveries were remarkable because they indicated that two characteristics of the gastrointestinal tract, high CO_2 and GlcNAc, both products of the enormous bacterial microbiota, signaled *MTL*-homozygous strains of *C. albicans* to switch and mate. In addition, glucose was shown to be a minor inducer of switching. The CO_2, GlcNAc, and glucose results were combined by Huang et al. (38) into a model for the regulation of switching by molecules found in the host gastrointestinal tract (Fig. 5), the major niche for *C. albicans* commensalism. Each extracellular signal is transduced by two pathways, one unidentified and the other the Ras1/cyclic AMP (cAMP) pathway.

THE REGULATION OF SWITCHING

The host signals that have been identified stimulate the rate of white-to-opaque switching, but switching occurs

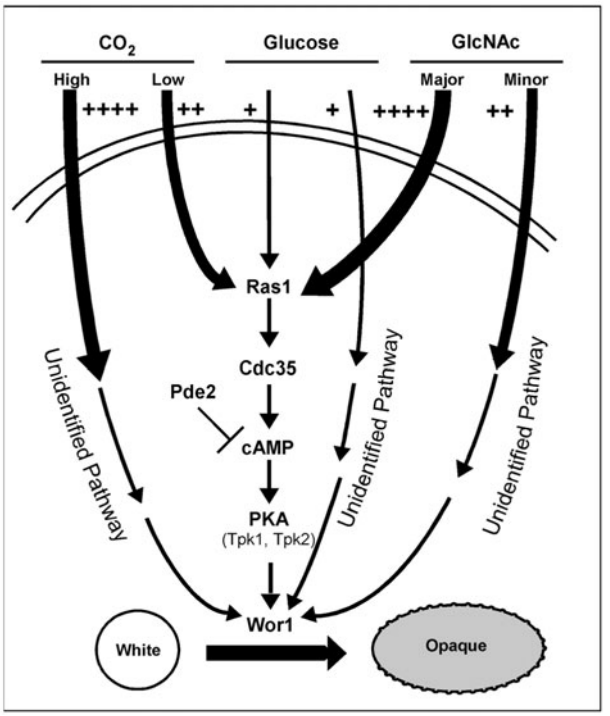

FIGURE 5 Spontaneous switching is regulated by at least three environmental components of the gastrointestinal tract, CO_2, glucose, and GlcNAc. Each function through the Ras1/cAMP pathway and an unidentified pathway. CO_2 and GlcNAc have one major and one minor pathway. The glucose effect is much lower than that of CO_2 and GlcNAc. All pathways function through Wor1. 10.1128/9781555817176.ch6f5

spontaneously and in both directions (4, 84, 96). What, then, are the molecular mechanisms within the cell that regulate spontaneous white-opaque switching? When white-opaque switching was first discovered by Slutsky et al. (96), the major examples we had for mechanisms of phenotypic switching in pathogens involved DNA reorganization. Bacteria switched between antigenic states by conserved rearrangements of DNA sequences containing upstream regulatory elements (14) or by reorganization in which one of many genes was moved to an expression site in the genome (6). Trypanosomes also moved genes from silenced to expressed regions of the genome (7). Another model, one that involved epigenetic changes at a master switch locus, was also proposed (98). This was based on the metastability of the expression of genes placed in subtelomeric regions in *S. cerevisiae* (5, 32, 76). Such genes switched in *S. cerevisiae* at approximately 1 in 1,000 cell divisions between an expressed and unexpressed state without DNA reorganization, but rather as a result of a positional effect. In the latter mechanism, it was argued that metastable changes in chromatin state were responsible for switching, and two studies lended weight to the application of this hypothesis to switching in *C. albicans*. It was demonstrated that either a histone deacetylase inhibitor or deletion of either of the deacetylase genes *HDA1* and *RPD3* dramatically affected the frequency of switching in one or both directions (46, 106). But these experiments provided only indirect proof. Direct proof of mechanism could be obtained only by identifying a master switch locus, if such a locus existed, and this occurred simultaneously in three laboratories in 2006. The master switch locus was referred to by one group (107) as *TOS9*, based on its homology to the ortholog *TOS9* in *S. cerevisiae*, and by the other two groups (36, 124) as *WOR1* (white-opaque regulator 1). The latter appears to have become the preferred name. By generating null mutants of six genes repressed by the **a**1-α2 corepressor complex in an **a**/**a** strain that did not express the complex, Zordan et al. (124) identified one mutant, *wor1*Δ, that did not switch. Huang et al. (36) screened for *C. albicans* genes that suppressed the *flo8* mutant phenotype in *S. cerevisiae*—i.e., a gene that reestablished invasiveness in agar. When they deleted the *C. albicans* gene *WOR1* identified by the screen, they found that **a**/**a** cells did not switch from white to opaque. Srikantha et al. (107) used a chromatin immunoprecipitation-on-chip analysis, developed at the time in Michael Snyder's laboratory for *C. albicans* (13), to identify binding sites for the **a**1-α2 corepressor complex, which had been shown to block switching. They found that of the identified genes, the transcription pattern of only one, *WOR1*, was consistent with that of a master switch gene. All three laboratories generated null mutants, and each laboratory performed both unique and common experiments to demonstrate that *WOR1* was the master switch locus.

The *WOR1* locus was fascinating for several reasons. First, it included an extremely long region upstream of the *WOR1* open reading frame of approximately 11,000 bp that contained five Wor1 binding sites at approximately bp -7900, -6100, 5100, -4100, and -2500, presumably for self-induction (124). Second, the transcript of *WOR1* contained a 5′ untranslated region of approximately 2,000 bp and was approximately 4,530 bp in length (107). In all three discovery papers, it was proposed that *WOR1* induced its own transcription, and the identification of five WOR1 binding sites by Zordan et al. (124) supported this basic model. Srikantha et al. (107) further proposed that the transcriptional regulation of *WOR1* also involved changes in chromatin state based on their initial observations on the roles of the chromatin deacetylase genes *HDA1* and RPD3 (106). Thus, in the publications of the *WOR1* discovery, it was proposed that a spontaneous switch from white to opaque was due to a stochastic increase in *WOR1* transcription above a threshold concentration, and a spontaneous switch from opaque to white was due to a stochastic decrease in *WOR1* transcription below a threshold concentration (36, 107, 124).

There were, however, indications in the literature that another transcription factor, Efg1 (52, 109), also regulated switching. Sonneborn et al. (103) reported that overexpression of *EFG1* resulted in a block in the white phase. Srikantha et al. (105) subsequently reported that both opaque- and white-phase cells expressed *EFG1* but that the transcription level in white-phase cells was far higher than in opaque-phase cells, and that the former resulted in a 3.2-kb transcript, whereas the latter resulted in a 2.2-kb transcript. Srikantha et al. (105) suggested a more complex role for *EFG1*, indicating that *EFG1* was not essential for switching, but rather for a subset of phenotypic changes necessary for full expression of the white phenotype. Zordan et al. (125), however, subsequently demonstrated that *EFG1* as well as *CZF1* and *WOR2* possessed *WOR1* binding sites, indicating that Wor1 regulated transcription of *EFG1*, *CZF1*, and *WOR2*. By analyzing a number of single and double mutants, they developed a model of regulation that involved a transcriptional feedback loop for both positive and negative regulation. In this model, *WOR1* positively regulated itself, *WOR2*, and *CZF1* and negatively regulated *EFG1*; *WOR2*

positively regulated *WOR1*; and *CZF1* negatively regulated *EFG1*. Vinces and Kumamato (117) demonstrated that Czf1 bound to its own promoter and that of *EFG1*, suggesting autoregulation of *CZF1* and regulation of *EFG1*. Zordan et al. (125) suggested in their model for the regulation of spontaneous white-opaque switching that in the white phase, expression of *EFG1* induced the white phenotype and repressed *WOR2*, *CZF1*, and *WOR1*, which was then expressed at levels below thresholds. In the opaque phase, expression of *WOR1* induced the opaque phenotype by inducing *CZF1*, which repressed *EFG1*, induced itself, and induced *WOR2*, which also induced *WOR1*. Hence, Zordan et al. (125) concluded that in the simplest form of the model, Efg1 was responsible for the white phenotype and Wor1 for the opaque phenotype, presumably by upregulating phase-specific genes. However, the complex phenotype of the *efg1*Δ mutant described by Srikantha et al. (105) must still be resolved in relation to the model. In addition, Huang et al. (38) have shown that Wor1 contains a Pka phosphorylation site and that Wor1 must be in the phosphorylated state for maximum stimulation of the white-to-opaque transition by GlcNAc, raising the issue of posttranscriptional regulation. Srikantha et al. (107) tagged Wor1 with green fluorescent protein and found that it reaccumulated in the nuclei of opaque cells but was undetectable in white cells. They found that when opaque cells converted to white cells, Wor1 was degraded.

Finally, Hnisz et al. (35) demonstrated that the methylation status of histone H3 at lysine 4 affected switching through the activity of the Set3/Hos2 histone deacetylase complex, adding weight to the original findings (46, 106) that the chromatin state of *WOR1* represented a meaningful component in the regulation of spontaneous switching, as suggested by Srikantha et al. (107).

THE ROLE OF SWITCHING IN MATING

The discovery that *MTL*-homozygous cells had to switch from white to opaque to mate (62) raised a more fundamental question. Why did *C. albicans* and the closely related species *C. dubliniensis* have to switch to opaque in order to mate? And why didn't any of the species other than *C. dubliniensis* in the *Candida* or *Saccharomyces* group of the hemiascomycetes have the same or similar requirement? Apparently, the white-opaque transition evolved in the ancestor of *C. albicans* and *C. dubliniensis*, the two of which diverged approximately 20 million years ago (63). Daniels et al. (22) appeared to find an answer to this question when they discovered that the mating pheromone, which is produced exclusively by opaque cells, also affects white cells by inducing them to become adhesive and enhancing the thickness of the basic biofilm formed by white cells. They hypothesized that minority opaque cells induced white cells of the opposite mating type to form a white cell biofilm that facilitated mating of the minority opaque cells, by stabilizing the pheromone gradients that directed **a/a** and α/α conjugation tubes, through the process of chemotropism, to find each other and fuse (Fig. 6) (22). Gradients of pheromone would be extremely prone to mechanical disruption and dissipation by diffusion if in a purely liquid environment. A biofilm would provide a protective environment against disruption, and a porous matrix would reduce diffusion. Daniels et al. (22) tested this hypothesis and demonstrated that α-pheromone and **a**-pheromone induced **a/a** and α/α white cells, respectively, to become more cohesive, become more adhesive to a substrate, and form biofilms that were

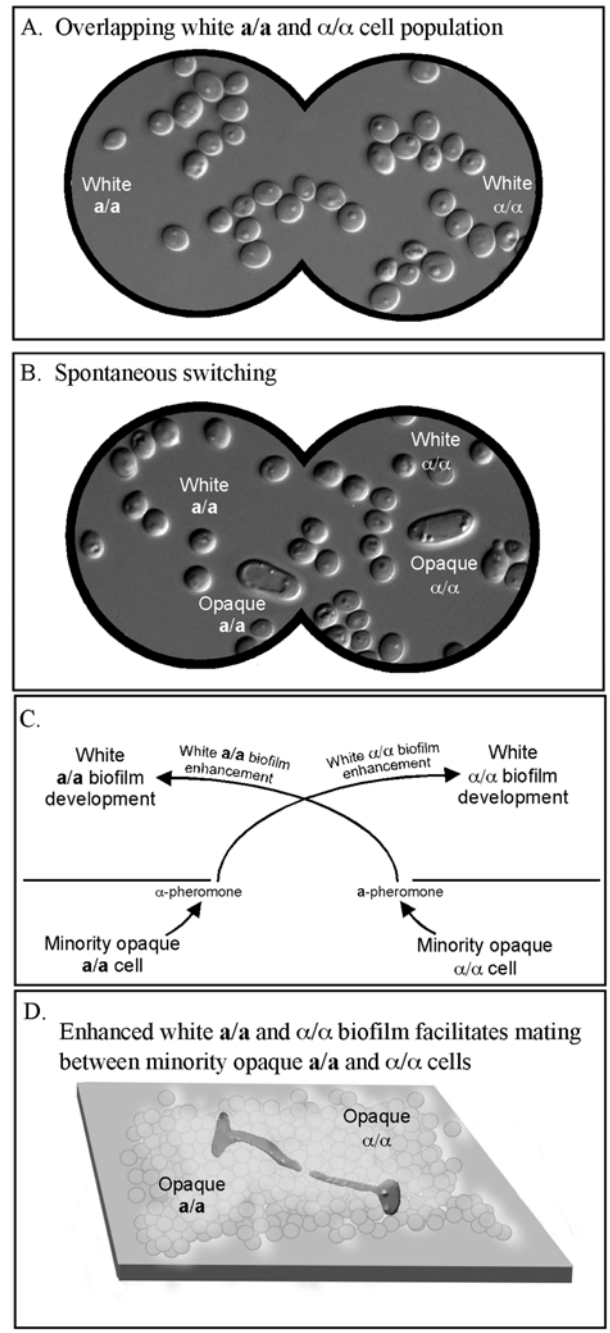

FIGURE 6 It has been hypothesized (22) that the white cell response to pheromone is to facilitate mating by developing an enhanced biofilm which serves as a protective environment for opaque cell mating. The hypothesized steps are as follows. (A) White **a/a** and α/α cells overlap in nature. (B) Rare opaque **a/a** and α/α cells appear as a result of spontaneous switching. (C) These minority opaque cells induce white cells of opposite mating type to form a biofilm. (D) Biofilms protect pheromone gradients while driving chemotropism in the mating process.

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30 to 50% thicker than the basic ones formed in the absence of pheromone of the opposite mating type. Finally, they demonstrated that by seeding white cell biofilms with vitally stained opaque **a/a** and α/α cells, the white cell biofilm facilitated chemotropism (22).

Skepticism of the validity of the white cell response was voiced by Bennett and Johnson (11) in a microarray comparison of α-pheromone-induced gene expression in white cells of two strains, the natural **a/a** strain P37005 (53) and a laboratory strain derived from SC5314 (8). They found a far weaker pheromone response in the latter laboratory strain and voiced concern that the white response to pheromone might be strain specific and medium dependent. Sahni et al. (90) responded to this skepticism by demonstrating that the white response was perfectly intact in 22 related strains, including laboratory derivatives of SC5314, thus laying to rest any doubt of its validity and importance.

THE REGULATION OF THE WHITE CELL PHEROMONE RESPONSE

Lockhart et al. (55) first demonstrated that α-pheromone upregulated three mating-related genes in white cells, *CAG1*, *STE4*, and *STE2*, which were also upregulated by α-pheromone in opaque cells. Daniels et al. (22) then showed that α-pheromone upregulated *CEK1*, *CEK2*, and *SST2*, also upregulated in opaque cells, but not *FIG1* or *KAR4*, two additional genes upregulated in opaque cells. Sahni et al. (89) then showed that α-pheromone upregulated 12 genes selectively in **a/a** white but not **a/a** opaque cells (*EAP1*, *PGA10*, *CSH1*, *PBR1*, *RBT5*, *LSP1*, *PHR1*, *PHR2*, *SUN41*, *WH11*, Orf19.207, and *CIT1*). The results demonstrated that in the white cell response to pheromone, genes are upregulated in white cells that are not upregulated by pheromone in opaque cells.

But which signal transduction pathways regulated the response? By mutational analysis, Yi et al. (121) first demonstrated that the same upstream components of the opaque cell pheromone response pathway were used in the white cell pheromone response pathway. This included the receptors, Ste2 and Ste3, a component of the same trimeric G protein complex, Ste4, and the last components of the same MAP kinase cascade, Cek1 and Cek2 (Fig. 4) (8, 18, 59, 121). Yi et al. (121) further showed that Far1 played no role in the white cell pheromone response, in contrast to its role in the opaque cell response. Sahni et al. (91) then showed that although the same signals, receptors, trimeric G protein complex, and MAP kinase cascade were shared by the opaque and white cell pheromone responses, the targeted transcription factors differed. Whereas Cph1, encoded by an ortholog of *STE12* in *S. cerevisiae*, was the transcription factor targeted by the MAP kinase cascade in the opaque response (8, 18, 59), Tec1 was the factor targeted in the white response (Fig. 4) (91). As would be expected, *CPH1* is upregulated by pheromone in the opaque but not white response, and *TEC1* is upregulated by pheromone in the white but not opaque response.

Sahni et al. (89) searched for a consensus sequence in the promoters of genes selectively upregulated in white cells and a consensus sequence in the promoters of genes selectively upregulated in opaque cells. They found an A-rich white-specific region with the consensus sequence AAAAAAAAAAGAAAG and a G-rich opaque-specific region with the consensus sequence GTGAGGGGA. The promoters of genes upregulated in both white and opaque cells by pheromone contained one copy of each of the respective consensus sequences. By deletion analysis, Sahni et al. (89) demonstrated that these regions, which they named WPRE and OPRE, respectively, did serve as the *cis*-acting activation sites for pheromone-induced expression of white- and opaque-specific genes, respectively. Interestingly, six bases at one end of WPRE were homologous to the Tec1 binding site, TCS, in the promoters of genes regulated by that factor in *S. cerevisiae* (91).

EVOLUTION OF THE WHITE CELL PHEROMONE RESPONSE PATHWAY

It is clear from the homologies between *C. albicans* and *S. cerevisiae* that the pheromone response pathway in the mating process has been highly conserved throughout the hemiascomycetes. Every component from pheromone throughout the trimeric G protein complex and MAP kinase cascade, as well as the targeted transcription factor, has been conserved between the two species. However, only the upper portion of the white cell pheromone response pathway is homologous to that of the pheromone response pathway of *S. cerevisiae* **a** or α cells and the pheromone response pathway of *C. albicans* **a/a** or α/α opaque cells (Fig. 7A). Tec1 appears to have been derived from an ancestral filamentation pathway (Fig. 7B), since it appears that it still plays a role in filamentation in both *C. albicans* and *S. cerevisiae* (57, 73, 95), and the target genes appear to have been derived from an ancestral biofilm program (Fig. 7C) (91). Hence, the white pheromone response pathway appears to be relatively new, only appearing in the ancestor to *C. albicans* and *C. dubliniensis*, and deriving all of its components from three ancestral programs (Fig. 7). Interestingly, this pathway provides unique insights into how signal transduction pathways evolve in alternative cell types (91).

Because the white pheromone response pathway is relatively young and has been derived from other ancestral pathways and processes still functioning in *C. albicans*, it probably has not had time to evolve further by changing components or functions. But there is one sign that suggests components are evolving. Yi et al. (122) identified a 55-amino-acid sequence in the first internal loop, IC1, of the α-pheromone receptor Ste2, which was missing from the IC1 loop of the highly homologous *S. cerevisiae* Ste2. When this region was deleted, it was found that the receptor did not function in the white pheromone response but did in the opaque pheromone response. Hence, the receptor common to the white and opaque response has evolved to serve a selective function for the white pheromone response. The role of the 55-amino-acid region in the selective activation of the white response has not been elucidated.

SWITCHING AND MATING IN *C. DUBLINIENSIS*

C. albicans and *C. dubliniensis* diverged as separate species some 20 million years ago (63) but still share a large number of traits (110, 111). The two species differ by an estimated 5% of genes based on comparative genome hybridization (64). Another major difference is the extraordinary genomic instability of *C. dubliniensis*, reflected in the rapid changes observed in the electrophoretic karyotypes of single strains and the increased variability between strains compared to *C. albicans* (43, 60). This genomic instability, which is due to an elevated frequency of recombination, has been suggested to be both favorable and detrimental! It can be favorable for rapid adaptation, as in the case of fluconazole resistance (65), or it can be detrimental, resulting in the degeneration of developmental programs, as is the case for hypha formation (108), white-opaque switching (79), and

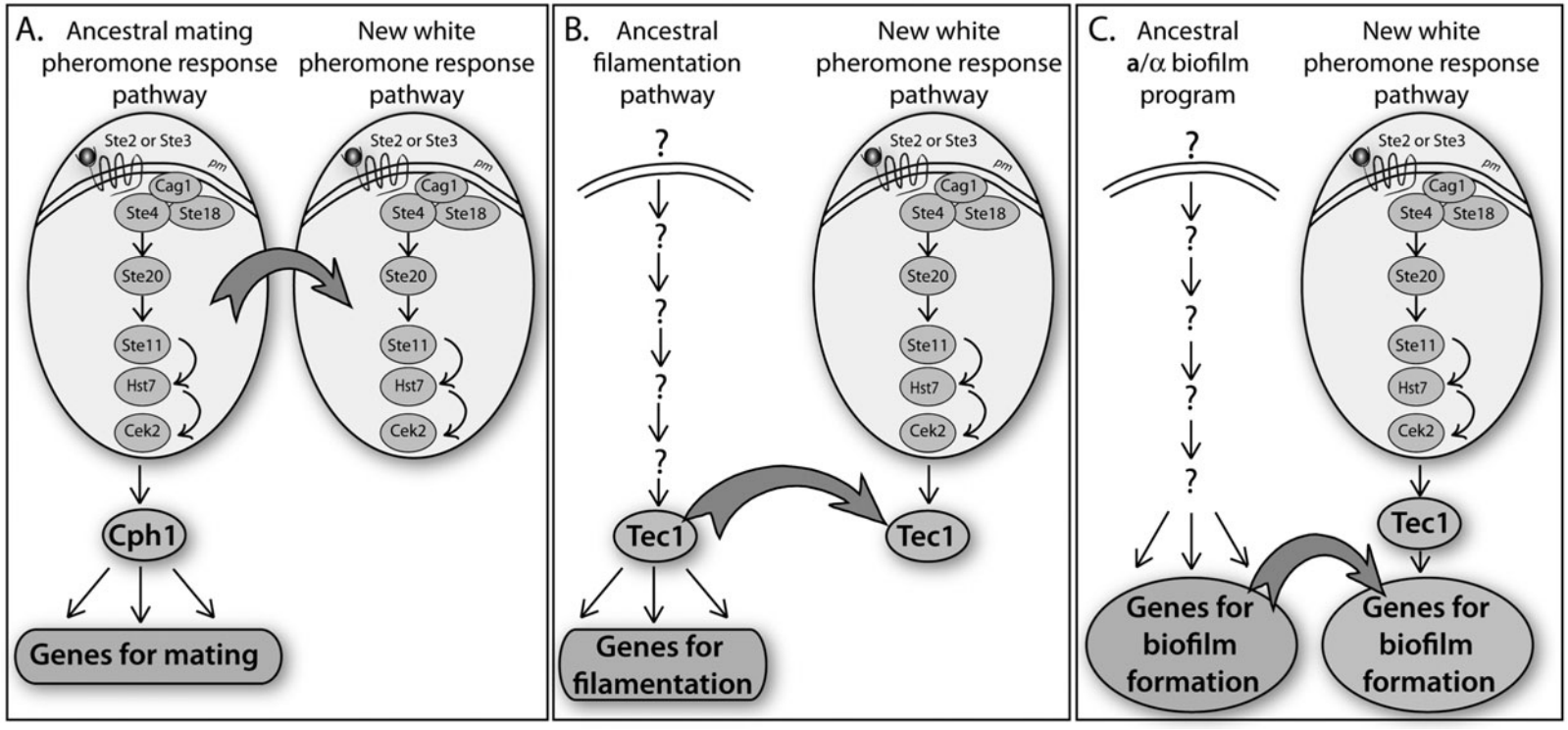

FIGURE 7 Hypothesized evolution of the white pheromone response pathway. Note that all components were derived from ancestral pathways still active in *C. albicans*. (A) The upper components were all derived from the ancestral pathways for the pheromone response of mating competent cells to pheromone. (B) The transcription factor Tec1 was derived from a filamentation pathway. (C) The downstream genes upregulated by Tec1 were derived from the biofilm program. See the work of Sahni et al. (91) for details. 10.1128/9781555817176.ch6f7

mating (79). *C. dubliniensis*, like *C. albicans*, undergoes white-opaque switching, but it forms strange opaque colony phenotypes, exhibits high rates of reversibility from opaque to white, and exhibits aberrant opaque cellular phenotypes in multiple strains (79). Even so, the unique pimpled surface of opaque cells, even aberrantly shaped ones, is still present (79).

C. dubliniensis is diploid, like *C. albicans*, and possesses a mating-type locus highly similar to that of *C. albicans* (79). However, the proportion of natural strains that are **a**/α is far lower than that of *C. albicans*, 67% versus approximately 90% (79). Remarkably, **a**/**a** and α/α strains of *C. dubliniensis* not only mate but also mate with *C. albicans*. They mate at higher frequencies interspecifically than intraspecifically in liquid cultures (79). When *C. dublinienis* mates with *C. albicans*, it undergoes all of the steps in nuclear fusion and daughter cell formation observed for *C. albicans* (79). Finally, *C. dubliniensis* mates on skin with cells of opposite mating type of its own species and of *C. albicans* with high efficiency. Together these results suggest that although *C. dubliniensis* appears to have the capacity to generate drug-resistant strains at higher frequencies then *C. albicans*, it probably does so through its high level of genetic instability, which also leads to the deterioration of developmental progress such as switching and mating.

QUESTIONS UNANSWERED

There still exist many unanswered questions related to switching and mating in *C. albicans*. In the case of mating, we still do not know the roles of the three genes, *OBP*, *PIK*, and *PAP*, embedded in the *MTL* locus. These genes are embedded in the *MTL* locus of species throughout the *Candida* group of the hemiascomycetes, but not the *Saccharomyces* group (17). Two of these genes, *PIK* and *PAP*, are essential, which may play a role in the maintenance of the locus, if selection pressure resulting from mating is weak (70). The **a** and α alleles of the three embedded genes are far more divergent than those of genes outside the *MTL* locus, which may have evolved different functions, as recently suggested for the *PAP* alleles (61). The observation that *MTL* zygosity may provide an advantage to **a**/α cells in the mouse model may be related to the divergence of these genes. That argument can also be applied to the mating-type genes, especially **a**1 and α2, which encode proteins in the **a**1-α2 corepressor complex. It may be that this corepressor not only blocks mating and switching but also affects genes involved in pathogenesis.

The observation that opaque cells of the same mating type self-mate at low frequencies in vitro is also an extremely interesting observation (1). This process may play an important role in generating diversity, although there may be a requirement for deleting *BAR1*, which encodes a protease that degrades α-pheromone. This phenomenon deserves more attention.

Although meiosis has not been observed, it has been searched for, primarily in vitro. Forche and coworkers (28) have demonstrated that tetraploids, the result of mating, return to the diploid or near-diploid state by the random loss of chromosomes and that this relatively unpredictable process involves recombination and the *SPO11* gene, which is essential for recombination. Yet, many of the meiosis genes, the functions of which have been elucidated in *S. cerevisiae*, remain intact in *C. albicans* (115). Could it be that meiosis is indeed functional in *C. albicans*, but the starvation conditions that induce meiosis in *S. cerevisiae* don't work for *C. albicans*? Could it be that meiosis is tied to a particular host niche or condition? The search for meiosis should not be abandoned. Remember, no one believed that *C. albicans* had a sexual cycle until the discovery of the *MTL* locus in 1999 (40). In addition, Reedy et al. (82) have demonstrated that meiosis occurs in *Candida lusitaniae*, a species in the *Candida* group of the hemiascomycetes that lacks even more of the meiotic genes established in *S. cerevisiae* than *C. albicans* lacks (102).

And why do *C. albicans* and *C. dubliniensis* have to undergo a phenotypic switch from white to opaque in order to mate? It has only been clearly demonstrated in vitro that a unique signaling system has evolved between white and opaque in which the former, through the release of pheromone, signals the latter to become adhesive, forming an enhanced biofilm that facilitates mating (22).

And why is the white cell pheromone response so robust and intact in strains of *C. albicans* from all of the major clades? Sahni et al. (90) demonstrated this to be the case in 27 tested strains, including **a**/**a** and α/α strains as well as genetically altered strains from the original laboratory strain, SC5314. Indeed, the white pheromone response seems to have been better conserved within the species than the opaque cell pheromone response in the mating process. This is also true for the species *C. dubliniensis* (C. Pujol, N. Sahni, S. Yi, and D. R. Soll, unpublished data). Could the white response to pheromone play a role in pathogenesis that is under greater selective pressure than the opaque mating response? If such a role was elucidated, it could potentially provide us with new drug targets.

Finally, why has the mating process been so carefully preserved in *C. albicans*, when it has clearly been demonstrated that mating and recombination are rare events both among strains of different clades and among strains of the same clade (15, 27, 33, 72, 78, 80, 112)? Could it be that by incorporating switching and mating into a pathogenic trait under high selective pressure, like biofilm formation, the mating system has been protected for the rare occasion when it is needed? Hopefully, this chapter will serve not only as an important resource but also as a stimulant for new experiments that drive this area of *C. albicans* research forward.

I am indebted to Nidhi Sahni, Song Yi, and Claude Pujol for editing the manuscript and to Karla Daniels for generating the figures.

REFERENCES

1. **Alby, K., D. Schaefer, and R. J. Bennett.** 2009. Homothallic and heterothallic mating in the opportunistic pathogen *Candida albicans*. *Nature* **460:**890–893.
2. **Anderson, J., L. Cundiff, B. Schnars, M. X. Gao, I. Mackenzie, and D. R. Soll.** 1989. Hypha formation in the white-opaque transition of *Candida albicans*. *Infect. Immun.* **57:**458–467.
3. **Anderson, J., R. Mihalik, and D. R. Soll.** 1990. Ultrastructure and antigenicity of the unique cell wall pimple of the *Candida* opaque phenotype. *J. Bacteriol.* **172:**224–235.
4. **Anderson, J. M., and D. R. Soll.** 1987. Unique phenotype of opaque cells in the white-opaque transition of *Candida albicans*. *J. Bacteriol.* **169:**5579–5588.
5. **Aparicio, O. M., B. L. Billington, and D. E. Gottschling.** 1991. Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. *Cell* **66:**1279–1287.
6. **Barbour, A. G.** 2003. Antigenic variation in *Borrelia*: relapsing fever and Lyme borreliosis, p. 319–356. In A. Craig

and A. Scherf (ed.), *Antigenic Variation*. Academic Press Elsevier, London, United Kingdom.

7. **Barry, J. D., and R. McCulloch.** 2003. Trypanosome antigenic variation—a heavy investment in the evasion of immunity, p. 224–242. *In* A. Craig and A. Scherf (ed.), *Antigenic Variation*. Academic Press Elsevier, London, United Kingdom.
8. **Bennett, R. J., M. A. Uhl, M. G. Miller, and A. D. Johnson.** 2003. Identification and characterization of a *Candida albicans* mating pheromone. *Mol. Cell. Biol.* **23:**8189–8201.
9. **Bennett, R. J., M. G. Miller, P. R. Chua, M. E. Maxon, and A. D. Johnson.** 2005. Nuclear fusion occurs during mating in *Candida albicans* and is dependent on the KAR3 gene. *Mol. Microbiol.* **55:**1046–1059.
10. **Bennett, R. J., and A. D. Johnson.** 2003. Completion of a parasexual cycle in *Candida albicans* by induced chromosome loss in tetraploid strains. *EMBO J.* **22:**2505–2515.
11. **Bennett, R. J., and A. D. Johnson.** 2006. The role of nutrient regulation and the Gpa2 protein in the mating pheromone response of *C. albicans*. *Mol. Microbiol.* **62:** 100–119.
12. **Bergen, M. S., E. Voss, and D. R. Soll.** 1990. Switching at the cellular level in the white-opaque transition of *Candida albicans*. *J. Gen. Microbiol.* **136:**1925–1936.
13. **Borneman, A. R., T. A. Gianoulis, Z. D. Zhang, H. Yu, J. Rozowsky, M. R. Seringhaus, L. Y. Wang, M. Gerstein, and M. Snyder.** 2007. Divergence of transcription factor binding sites across related yeast species. *Science* **317:**815–819.
14. **Borst, P. 2003.** Mechanisms of antigenic variation: an overview, p. 1–15. *In* A. Craig and A. Scherf (ed.), *Antigenic Variation*. Academic Press Elsevier, London, United Kingdom.
15. **Bougnoux, M. E., C. Pujol, D. Diogo, C. Bouchier, D. R. Soll, and C. d'Enfert.** 2008. Mating is rare within as well as between clades of the human pathogen *Candida albicans*. *Fungal Genet. Biol.* **45:**221–231.
16. **Braun, B. R., H. M. van Het, C. d'Enfert, M. Martchenko, J. Dungan, A. Kuo, D. O. Inglis, M. A. Uhl, H. Hogues, M. Berriman, M. Lorenz, A. Levitin, U. Oberholzer, C. Bachewich, D. Harcus, A. Marcil, D. Dignard, T. Iouk, R. Zito, L. Frangeul, F. Tekaia, K. Rutherford, E. Wang, C. A. Munro, S. Bates, N. A. Gow, L. L. Hoyer, G. Kohler, J. Morschhauser, G. Newport, S. Znaidi, M. Raymond, B. Turcotte, G. Sherlock, M. Costanzo, J. Ihmels, J. Berman, D. Sanglard, N. Agabian, A. P. Mitchell, A. D. Johnson, M. Whiteway, and A. Nantel.** 2005. A human-curated annotation of the *Candida albicans* genome. *PLoS Genet.* **1:**36–57.
17. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J. L. Reedy, I. Agrafioti, M. B. Arnaud, S. Bates, A. J. Brown, S. Brunke, M. C. Costanzo, D. A. Fitzpatrick, P. W. de Groot, D. Harris, L. L. Hoyer, B. Hube, F. M. Klis, C. Kodira, N. Lennard, M. E. Logue, R. Martin, A. M. Neiman, E. Nikolaou, M. A. Quail, J. Quinn, M. C. Santos, F. F. Schmitzberger, G. Sherlock, P. Shah, K. A. Silverstein, M. S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M. P. Stumpf, P. E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N. A. Gow, M. C. Lorenz, B. W. Birren, M. Kellis, and C. A. Cuomo.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459:**657–662.
18. **Chen, J., J. Chen, S. Lane, and H. Liu.** 2002. A conserved mitogen-activated protein kinase pathway is required for mating in *Candida albicans*. *Mol. Microbiol.* **46:**1335–1344.
19. **Chen, P., S. K. Sapperstein, J. D. Choi, and S. Michaelis.** 1997. Biogenesis of the *Saccharomyces cerevisiae* mating pheromone **a**-factor. *J. Cell Biol.* **136:**251–269.
20. **Cherkasova, V., D. M. Lyons, and E. A. Elion.** 1999. Fus3p and Kss1p control G1 arrest in *Saccharomyces cerevisiae* through a balance of distinct arrest and proliferative functions that operate in parallel with Far1p. *Genetics* **151:**989–1004.
21. **Cote, P., and M. Whiteway.** 2008. The role of *Candida albicans* FAR1 in regulation of pheromone-mediated mating, gene expression and cell cycle arrest. *Mol. Microbiol.* **68:**392–404.
22. **Daniels, K. J., T. Srikantha, S. R. Lockhart, C. Pujol, and D. R. Soll.** 2006. Opaque cells signal white cells to form biofilms in *Candida albicans*. *EMBO J.* **25:** 2240–2252.
23. **Dignard, D., and M. Whiteway.** 2006. SST2, a regulator of G-protein signaling for the *Candida albicans* mating response pathway. *Eukaryot. Cell* **5:**192–202.
24. **Dignard, D., A. L. El-Naggar, M. E. Logue, G. Butler, and M. Whiteway.** 2007. Identification and characterization of MFA1, the gene encoding *Candida albicans* **a**-factor pheromone. *Eukaryot. Cell* **6:**487–494.
25. **Dranginis, A. M.** 1990. Binding of yeast a1 and alpha 2 as a heterodimer to the operator DNA of a haploid-specific gene. *Nature* **347:**682–685.
26. **Dumitru, R., D. H. Navarathna, C. P. Semighini, C. G. Elowsky, R. V. Dumitru, D. Dignard, M. Whiteway, A. L. Atkin, and K. W. Nickerson.** 2007. In vivo and in vitro anaerobic mating in *Candida albicans*. *Eukaryot. Cell* **6:**465–472.
27. **Forche, A., G. Schonian, Y. Graser, R. Vilgalys, and T. G. Mitchell.** 1999. Genetic structure of typical and atypical populations of *Candida albicans* from Africa. *Fungal Genet. Biol.* **28:**107–125.
28. **Forche, A., K. Alby, D. Schaefer, A. D. Johnson, J. Berman, and R. J. Bennett.** 2008. The parasexual cycle in *Candida albicans* provides an alternative pathway to meiosis for the formation of recombinant strains. *PLoS Biol.* **6:**e110.
29. **Gartner, A., A. Jovanović, D.-I. Jeoung, S. Bourlat, F. R. Cross, and G. Ammerer.** 1998. Pheromone-dependent G_1 cell cycle arrest requires Far1 phosphorylation, but may not involve inhibition of Cdc28-Cln2 kinase, in vivo. *Mol. Cell. Biol.* **18:**3681–3691.
30. **Geiger, J., D. Wessels, S. R. Lockhart, and D. R. Soll.** 2004. Release of a potent polymorphonuclear leukocyte chemoattractant is regulated by white-opaque switching in *Candida albicans*. *Infect. Immun.* **72:**667–677.
31. **Goldman, G. H., M. E. da Silva Ferreira, M. E. dos Reis, M. Savoldi, D. Perlin, S. Park, P. C. Godoy Martinez, M. H. Goldman, and A. L. Colombo.** 2004. Evaluation of fluconazole resistance mechanisms in *Candida albicans* clinical isolates from HIV-infected patients in Brazil. *Diagn. Microbiol. Infect. Dis.* **50:**25–32.
32. **Gottschling, D. E., O. M. Aparicio, B. L. Billington, and V. A. Zakian.** 1990. Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell* **63:**751–762.
33. **Graser, Y., M. Volovsek, J. Arrington, G. Schonian, W. Presber, T. G. Mitchell, and R. Vilgalys.** 1996. Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. *Proc. Natl. Acad. Sci. USA* **93:**12473–12477.
34. **Heitman, J.** 2009. Microbial genetics: love the one you're with. *Nature* **460:**807–808.
35. **Hnisz, D., T. Schwarzmuller, and K. Kuchler.** 2009. Transcriptional loops meet chromatin: a dual-layer network

controls white-opaque switching in *Candida albicans*. *Mol. Microbiol.* **74:**1–15.
36. **Huang, G., H. Wang, S. Chou, X. Nie, J. Chen, and H. Liu.** 2006. Bistable expression of WOR1, a master regulator of white-opaque switching in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **103:**12813–12818.
37. **Huang, G., T. Srikantha, N. Sahni, S. Yi, and D. R. Soll.** 2009. CO_2 regulates white-to-opaque switching in *Candida albicans*. *Curr. Biol.* **19:**330–334.
38. **Huang, G., S. Yi, N. Sahni, K. J. Daniels, T. Srikantha, and D. R. Soll.** 2010. *N*-Acetylglucosamine induces white to opaque switching, a mating prerequisite in *Candida albicans*. *PLoS Pathog.* **6:**e1000806.
39. **Hube, B., M. Monod, D. A. Schofield, A. J. Brown, and N. A. Gow.** 1994. Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. *Mol. Microbiol.* **14:**87–99.
40. **Hull, C. M., and A. D. Johnson.** 1999. Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. *Science* **285:**1271–1275.
41. **Hull, C. M., R. M. Raisner, and A. D. Johnson.** 2000. Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. *Science* **289:**307–310.
42. **Ibrahim, A. S., B. B. Magee, D. C. Sheppard, M. Yang, S. Kauffman, J. Becker, J. E. Edwards, Jr., and P. T. Magee.** 2005. Effects of ploidy and mating type on virulence of *Candida albicans*. *Infect. Immun.* **73:**7366–7374.
43. **Joly, S., C. Pujol, and D. R. Soll.** 2002. Microevolutionary changes and chromosomal translocations are more frequent at RPS loci in *Candida dubliniensis* than in *Candida albicans*. *Infect. Genet. Evol.* **2:**19–37.
44. **Jones, T., N. A. Federspiel, H. Chibana, J. Dungan, S. Kalman, B. B. Magee, G. Newport, Y. R. Thorstenson, N. Agabian, P. T. Magee, R. W. Davis, and S. Scherer.** 2004. The diploid genome sequence of *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **101:**7329–7334.
45. **Kennedy, M. J., A. L. Rogers, L. R. Hanselmen, D. R. Soll, and R. J. Yancey, Jr.** 1988. Variation in adhesion and cell surface hydrophobicity in *Candida albicans* white and opaque phenotypes. *Mycopathologia* **102:**149–156.
46. **Klar, A. J., T. Srikantha, and D. R. Soll.** 2001. A histone deacetylation inhibitor and mutant promote colony-type switching of the human pathogen *Candida albicans*. *Genetics* **158:**919–924.
47. **Kvaal, C., S. A. Lachke, T. Srikantha, K. Daniels, J. McCoy, and D. R. Soll.** 1999. Misexpression of the opaque-phase-specific gene PEP1 (SAP1) in the white phase of *Candida albicans* confers increased virulence in a mouse model of cutaneous infection. *Infect. Immun.* **67:**6652–6662.
48. **Kvaal, C. A., T. Srikantha, and D. R. Soll.** 1997. Misexpression of the white-phase-specific gene WH11 in the opaque phase of *Candida albicans* affects switching and virulence. *Infect. Immun.* **65:**4468–4475.
49. **Lachke, S. A., S. R. Lockhart, K. J. Daniels, and D. R. Soll.** 2003. Skin facilitates *Candida albicans* mating. *Infect. Immun.* **71:**4970–4976.
50. **Lan, C. Y., G. Newport, L. A. Murillo, T. Jones, S. Scherer, R. W. Davis, and N. Agabian.** 2002. Metabolic specialization associated with phenotypic switching in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **99:**14907–14912.
51. **Legrand, M., P. Lephart, A. Forche, F. M. Mueller, T. Walsh, P. T. Magee, and B. B. Magee.** 2004. Homozygosity at the MTL locus in clinical strains of *Candida albicans*: karyotypic rearrangements and tetraploid formation. *Mol. Microbiol.* **52:**1451–1462.
52. **Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90:**939–949.
53. **Lockhart, S. R., C. Pujol, K. J. Daniels, M. G. Miller, A. D. Johnson, M. A. Pfaller, and D. R. Soll.** 2002. In *Candida albicans*, white-opaque switchers are homozygous for mating type. *Genetics* **162:**737–745.
54. **Lockhart, S. R., K. J. Daniels, R. Zhao, D. Wessels, and D. R. Soll.** 2003. Cell biology of mating in *Candida albicans*. *Eukaryot. Cell* **2:**49–61.
55. **Lockhart, S. R., R. Zhao, K. J. Daniels, and D. R. Soll.** 2003. Alpha-pheromone-induced "shmooing" and gene regulation require white-opaque switching during *Candida albicans* mating. *Eukaryot. Cell* **2:**847–855.
56. **Lohse, M. B., and A. D. Johnson.** 2009. White-opaque switching in *Candida albicans*. *Curr. Opin. Microbiol.* **12:**650–654.
57. **Madhani, H. D., and G. R. Fink.** 1997. Combinatorial control required for the specificity of yeast MAPK signaling. *Science* **275:**1314–1317.
58. **Magee, B. B., and P. T. Magee.** 2000. Induction of mating in *Candida albicans* by construction of *MTL***a** and *MTL*α strains. *Science* **289:**310–313.
59. **Magee, B. B., M. Legrand, A. M. Alarco, M. Raymond, and P. T. Magee.** 2002. Many of the genes required for mating in *Saccharomyces cerevisiae* are also required for mating in *Candida albicans*. *Mol. Microbiol.* **46:**1345–1351.
60. **Magee, B. B., M. D. Sanchez, D. Saunders, D. Harris, M. Berriman, and P. T. Magee.** 2008. Extensive chromosome rearrangements distinguish the karyotype of the hypovirulent species *Candida dubliniensis* from the virulent *Candida albicans*. *Fungal Genet. Biol.* **45:**338–350.
61. **Manoharlal, R., J. Gorantala, M. Sharma, D. Sanglard, and R. Prasad.** 2010. PAP1 [poly(A) polymerase 1] homozygosity and hyperadenylation are major determinants of increased mRNA stability of *CDR1* in azole-resistant clinical isolates of *Candida albicans*. *Microbiology* **156:**313–326.
62. **Miller, M. G., and A. D. Johnson.** 2002. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell* **110:**293–302.
63. **Mishra, P. K., M. Baum, and J. Carbon.** 2007. Centromere size and position in *Candida albicans* are evolutionarily conserved independent of DNA sequence heterogeneity. *Mol. Genet. Genomics* **278:**455–465.
64. **Moran, G., C. Stokes, S. Thewes, B. Hube, D. C. Coleman, and D. Sullivan.** 2004. Comparative genomics using *Candida albicans* DNA microarrays reveals absence and divergence of virulence-associated genes in *Candida dubliniensis*. *Microbiology* **150:**3363–3382.
65. **Moran, G. P., D. J. Sullivan, M. C. Henman, C. E. McCreary, B. J. Harrington, D. B. Shanley, and D. C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. *Antimicrob. Agents Chemother.* **41:**617–623.
66. **Morrow, B., J. Anderson, J. Wilson, and D. R. Soll.** 1989. Bidirectional stimulation of the white-opaque transition of *Candida albicans* by ultraviolet irradiation. *J. Gen. Microbiol.* **135:**1201–1208.
67. **Morrow, B., T. Srikantha, and D. R. Soll.** 1992. Transcription of the gene for a pepsinogen, PEP1, is regulated by white-opaque switching in *Candida albicans*. *Mol. Cell. Biol.* **12:**2997–3005.
68. **Morrow, B., T. Srikantha, J. Anderson, and D. R. Soll.** 1993. Coordinate regulation of two opaque-phase-specific genes during white-opaque switching in *Candida albicans*. *Infect. Immun.* **61:**1823–1828.
69. **Nasmyth, K. A.** 1982. Molecular genetics of yeast mating type. *Annu. Rev. Genet.* **16:**439–500.

70. **Nielsen, K., and J. Heitman.** 2007. Sex and virulence of human pathogenic fungi. *Adv. Genet.* **57:**143–173.
71. **Odds, F. C.** 1987. *Candida* infection: an overview. *Crit. Rev. Microbiol.* **15:**1–5.
72. **Odds, F. C., M. E. Bougnoux, D. J. Shaw, J. M. Bain, A. D. Davidson, D. Diogo, M. D. Jacobsen, M. Lecomte, S. Y. Li, A. Tavanti, M. C. Maiden, N. A. Gow, and C. d'Enfert.** 2007. Molecular phylogenetics of *Candida albicans*. *Eukaryot. Cell* **6:**1041–1052.
73. **Oehlen, L., and F. R. Cross.** 1998. The mating factor response pathway regulates transcription of TEC1, a gene involved in pseudohyphal differentiation of *Saccharomyces cerevisiae*. *FEBS Lett.* **429:**83–88.
74. **Panwar, S. L., M. Legrand, D. Dignard, M. Whiteway, and P. T. Magee.** 2003. MFα1, the gene encoding the α mating pheromone of *Candida albicans*. *Eukaryot. Cell* **2:**1350–1360.
75. **Peter, M., A. Gartner, J. Horecka, G. Ammerer, and I. Herskowitz.** 1993. FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. *Cell* **73:**747–760.
76. **Pillus, L., and J. Rine.** 1989. Epigenetic inheritance of transcriptional states in *S. cerevisiae*. *Cell* **59:**637–647.
77. **Pujol, C., J. Reynes, F. Renaud, M. Raymond, M. Tibayrenc, F. J. Ayala, F. Janbon, M. Mallie, and J. M. Bastide.** 1993. The yeast *Candida albicans* has a clonal mode of reproduction in a population of infected human immunodeficiency virus-positive patients. *Proc. Natl. Acad. Sci. USA* **90:**9456–9459.
78. **Pujol, C., S. Joly, B. Nolan, T. Srikantha, and D. R. Soll.** 1999. Microevolutionary changes in Candida albicans identified by the complex Ca3 fingerprinting probe involve insertions and deletions of the full-length repetitive sequence RPS at specific genomic sites. Microbiology **145**(Pt. 10):2635–2646.
79. **Pujol, C., K. J. Daniels, S. R. Lockhart, T. Srikantha, J. B. Radke, J. Geiger, and D. R. Soll.** 2004. The closely related species *Candida albicans* and *Candida dubliniensis* can mate. *Eukaryot. Cell* **3:**1015–1027.
80. **Pujol, C., A. Dodgson, and D. R. Soll.** 2005. Population genetics of ascomycetes pathogenic to humans and animals, p. 149–188. *In* J. Xu (ed.), *Evolutionary Genetics of Fungi*. Horizon Scientific Press, Norfolk, United Kingdom.
81. **Ramirez-Zavala, B., O. Reuss, Y. N. Park, K. Ohlsen, and J. Morschhauser.** 2008. Environmental induction of white-opaque switching in *Candida albicans*. *PLoS Pathog.* **4:**e1000089.
82. **Reedy, J. L., A. M. Floyd, and J. Heitman.** 2009. Mechanistic plasticity of sexual reproduction and meiosis in the *Candida* pathogenic species complex. *Curr. Biol.* **19:**891–899.
83. **Remenyi, A., M. C. Good, R. P. Bhattacharyya, and W. A. Lim.** 2005. The role of docking interactions in mediating signaling input, output, and discrimination in the yeast MAPK network. *Mol. Cell* **20:**951–962.
84. **Rikkerink, E. H., B. B. Magee, and P. T. Magee.** 1988. Opaque-white phenotype transition: a programmed morphological transition in *Candida albicans*. *J. Bacteriol.* **170:**895–899.
85. **Roberts, C. J., B. Nelson, M. J. Marton, R. Stoughton, M. R. Meyer, H. A. Bennett, Y. D. He, H. Dai, W. L. Walker, T. R. Hughes, M. Tyers, C. Boone, and S. H. Friend.** 2000. Signaling and circuitry of multiple MAPK pathways revealed by a matrix of global gene expression profiles. *Science* **287:**873–880.
86. **Robles, J. C., L. Koreen, S. Park, and D. S. Perlin.** 2004. Multilocus sequence typing is a reliable alternative method to DNA fingerprinting for discriminating among strains of *Candida albicans*. *J. Clin. Microbiol.* **42:**2480–2488.
87. **Rustad, T. R., D. A. Stevens, M. A. Pfaller, and T. C. White.** 2002. Homozygosity at the Candida albicans MTL locus associated with azole resistance. Microbiology **148:**1061–1072.
88. **Sadhu, C., D. Hoekstra, M. J. McEachern, S. I. Reed, and J. B. Hicks.** 1992. A G-protein α subunit from asexual *Candida albicans* functions in the mating signal transduction pathway of *Saccharomyces cerevisiae* and is regulated by the **a**1-α2 repressor. *Mol. Cell. Biol.* **12:**1977–1985.
89. **Sahni, N., S. Yi, K. J. Daniels, T. Srikantha, C. Pujol, and D. R. Soll.** 2009. Genes selectively up-regulated by pheromone in white cells are involved in biofilm formation in *Candida albicans*. *PLoS Pathog.* **5:**e1000601.
90. **Sahni, N., S. Yi, C. Pujol, and D. R. Soll.** 2009. The white cell response to pheromone is a general characteristic of *Candida albicans* strains. *Eukaryot. Cell* **8:**251–256.
91. **Sahni, N., S. Yi, K. J. Daniels, G. Huang, T. Srikantha, and D. R. Soll.** 2010. Tec1 mediates the pheromone response of the white phenotype of *Candida albicans*: insights into the evolution of new signal transduction pathways. *PLoS Biol.* **8:**e1000363.
92. **Sánchez-Martínez, C., and J. Pérez-Martín.** 2002. Gpa2, a G-protein alpha subunit required for hyphal development in *Candida albicans*. *Eukaryot Cell* **1:**865–874.
93. **Schaefer, D., P. Cote, M. Whiteway, and R. J. Bennett.** 2007. Barrier activity in *Candida albicans* mediates pheromone degradation and promotes mating. *Eukaryot. Cell* **6:**907–918.
94. **Schwartz, M. A., and H. D. Madhani.** 2004. Principles of MAP kinase signaling specificity in *Saccharomyces cerevisiae*. *Annu. Rev. Genet.* **38:**725–748.
95. **Schweizer, A., S. Rupp, B. N. Taylor, M. Röllinghoff, and K. Schröppel.** 2000. The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans*. *Mol. Microbiol.* **38:**435–445.
96. **Slutsky, B., M. Staebell, J. Anderson, L. Risen, M. Pfaller, and D. R. Soll.** 1987. "White-opaque transition": a second high-frequency switching system in *Candida albicans*. *J. Bacteriol.* **169:**189–197.
97. **Soll, D. R., C. J. Langtimm, J. McDowell, J. Hicks, and R. Galask.** 1987. High-frequency switching in *Candida* strains isolated from vaginitis patients. *J. Clin. Microbiol.* **25:**1611–1622.
98. **Soll, D. R.** 1992. High-frequency switching in *Candida albicans*. *Clin. Microbiol. Rev.* **5:**183–203.
99. **Soll, D. R.** 1995. The use of computers in understanding how animal cells crawl. *Int. Rev. Cytol.* **163:**43–104.
100. **Soll, D. R.** 2002. *Candida* commensalism and virulence: the evolution of phenotypic plasticity. *Acta Trop.* **81:**101–110.
101. **Soll, D. R.** 2009. Why does *Candida albicans* switch? *FEMS Yeast Res.* **9:**973–989.
102. **Soll, D. R., C. Pujol, and T. Srikantha.** 2009. Sex: deviant mating in yeast. *Curr. Biol.* **19:**R509–R511.
103. **Sonneborn, A., B. Tebarth, and J. F. Ernst.** 1999. Control of white-opaque phenotypic switching in *Candida albicans* by the Efg1p morphogenetic regulator. *Infect. Immun.* **67:**4655–4660.
104. **Srikantha, T., and D. R. Soll.** 1993. A white-specific gene in the white-opaque switching system of *Candida albicans*. *Gene* **131:**53–60.
105. **Srikantha, T., L. K. Tsai, K. Daniels, and D. R. Soll.** 2000. EFG1 null mutants of *Candida albicans* switch but cannot express the complete phenotype of white-phase budding cells. *J. Bacteriol.* **182:**1580–1591.
106. **Srikantha, T., L. Tsai, K. Daniels, A. J. Klar, and D. R. Soll.** 2001. The histone deacetylase genes HDA1 and

RPD3 play distinct roles in regulation of high-frequency phenotypic switching in *Candida albicans*. *J. Bacteriol.* **183:**4614–4625.

107. **Srikantha, T., A. R. Borneman, K. J. Daniels, C. Pujol, W. Wu, M. R. Seringhaus, M. Gerstein, S. Yi, M. Snyder, and D. R. Soll.** 2006. TOS9 regulates white-opaque switching in *Candida albicans*. *Eukaryot. Cell* **5:**1674–1687.
108. **Stokes, C., G. P. Moran, M. J. Spiering, G. T. Cole, D. C. Coleman, and D. J. Sullivan.** 2007. Lower filamentation rates of Candida dubliniensis contribute to its lower virulence in comparison with *Candida albicans*. *Fungal Genet. Biol.* **44:**920–931.
109. **Stoldt, V. R., A. Sonneborn, C. E. Leuker, and J. F. Ernst.** 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J.* **16:**1982–1991.
110. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov.: phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141**(Pt. 7)**:**1507–1521.
111. **Sullivan, D. J., G. P. Moran, and D. C. Coleman.** 2005. *Candida dubliniensis*: ten years on. *FEMS Microbiol. Lett.* **253:**9–17.
112. **Tavanti, A., N. A. Gow, M. C. Maiden, F. C. Odds, and D. J. Shaw.** 2004. Genetic evidence for recombination in *Candida albicans* based on haplotype analysis. *Fungal Genet. Biol.* **41:**553–562.
113. **Tavanti, A., A. D. Davidson, M. J. Fordyce, N. A. Gow, M. C. Maiden, and F. C. Odds.** 2005. Population structure and properties of *Candida albicans*, as determined by multilocus sequence typing. *J. Clin. Microbiol.* **43:**5601–5613.
114. **Tsong, A. E., M. G. Miller, R. M. Raisner, and A. D. Johnson.** 2003. Evolution of a combinatorial transcriptional circuit: a case study in yeasts. *Cell* **115:**389–399.
115. **Tzung, K. W., R. M. Williams, S. Scherer, N. Federspiel, T. Jones, N. Hansen, V. Bivolarevic, L. Huizar, C. Komp, R. Surzycki, R. Tamse, R. W. Davis, and N. Agabian.** 2001. Genomic evidence for a complete sexual cycle in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **98:**3249–3253.
116. **Vargas, K., P. W. Wertz, D. Drake, B. Morrow, and D. R. Soll.** 1994. Differences in adhesion of *Candida albicans* 3153A cells exhibiting switch phenotypes to buccal epithelium and stratum corneum. *Infect. Immun.* **62:**1328–1335.
117. **Vinces, M. D., and C. A. Kumamoto.** 2007. The morphogenetic regulator Czf1p is a DNA-binding protein that regulates white opaque switching in *Candida albicans*. *Microbiology* **153:**2877–2884.
118. **White, T. C., S. H. Miyasaki, and N. Agabian.** 1993. Three distinct secreted aspartyl proteinases in *Candida albicans*. *J. Bacteriol.* **175:**6126–6133.
119. **Wu, W., C. Pujol, S. R. Lockhart, and D. R. Soll.** 2005. Chromosome loss followed by duplication is the major mechanism of spontaneous mating-type locus homozygosis in *Candida albicans*. *Genetics* **169:**1311–1327.
120. **Wu, W., S. R. Lockhart, C. Pujol, T. Srikantha, and D. R. Soll.** 2007. Heterozygosity of genes on the sex chromosome regulates *Candida albicans* virulence. *Mol. Microbiol.* **64:**1587–1604.
121. **Yi, S., N. Sahni, K. J. Daniels, C. Pujol, T. Srikantha, and D. R. Soll.** 2008. The same receptor, G protein, and mitogen-activated protein kinase pathway activate different downstream regulators in the alternative white and opaque pheromone responses of *Candida albicans*. *Mol. Biol. Cell* **19:**957–970.
122. **Yi, S., N. Sahni, C. Pujol, K. J. Daniels, T. Srikantha, N. Ma, and D. R. Soll.** 2009. A *Candida albicans*-specific region of the alpha-pheromone receptor plays a selective role in the white cell pheromone response. *Mol. Microbiol.* **71:**925–947.
123. **Zhao, R., K. J. Daniels, S. R. Lockhart, K. M. Yeater, L. L. Hoyer, and D. R. Soll.** 2005. Unique aspects of gene expression during *Candida* albicans mating and possible G_1 dependency. *Eukaryot.Cell* **4:**1175–1190.
124. **Zordan, R. E., D. J. Galgoczy, and A. D. Johnson.** 2006. Epigenetic properties of white-opaque switching in *Candida albicans* are based on a self-sustaining transcriptional feedback loop. *Proc. Natl. Acad. Sci. USA* **103:**12807–12812.
125. **Zordan, R. E., M. G. Miller, D. J. Galgoczy, B. B. Tuch, and A. D. Johnson.** 2007. Interlocking transcriptional feedback loops control white-opaque switching in *Candida albicans*. *PLoS Biol.* **5:**e256.

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy
©2012 ASM Press, Washington, DC
10.1128/9781555817176.ch7

7

Detection and Clinical Significance of Variability among *Candida* Isolates

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Strain variability is a central topic in the discussion of *Candida* biology. Among the pathogenic species of *Candida*, variability has been examined most broadly for isolates of *Candida albicans*. For that reason, *C. albicans* receives the most attention in this chapter. Content in this chapter is parallel to other presentations in the text, most notably chapters discussing genome stability (chapter 5), mating (chapter 6), and clinical drug resistance (chapter 23). The reader is referred to these other chapters to gain a more complete understanding of the importance of strain variation to *Candida* biology.

ASSIGNMENT OF *CANDIDA* ISOLATES TO A SPECIES

Most of the *Candida* isolates that are studied are derived from clinical specimens. Therefore, a discussion of variability among *Candida* isolates within a particular species starts with decisions that are made in the diagnostic microbiology laboratory. Using available methods, the diagnostic microbiologist must accurately place each isolate into the proper taxonomic group. For the sake of this discussion, the assignment is to the species level. Incorrect assignment of an isolate leads to a larger estimate of variability within that species. Failure to recognize isolates as belonging to the same species leads to an unrealistically large number of species, each perhaps with an artificially narrow estimate of variability. Methods that are available for discrimination between species are derived from previous knowledge within the field. As that knowledge grows, the process of identifying clinical isolates becomes more refined.

Within the *Candida* field, initial characterization of clinical isolates involves colonial and cellular morphology. The presence or absence of certain morphological features, such as a germ tube or chlamydospores, provides clues to lead the diagnostician down the flowchart of possible identities for the isolate. Additional approaches, such as fermentation and assimilation tests, or assaying for the presence of a specific enzymatic function, can further refine isolate identification. These tests are often part of a commercial testing kit or automated identification system. More recently, molecular biology methods have become part of the diagnostic microbiologist's toolkit. Taxonomic decisions are driven by comparison of DNA sequences from genes encoding rRNA molecules, or of genes shown to provide very specific discrimination between closely related species. The recent description of *Candida subhashii* as a new species illustrates the methods used and decisions encountered in the process of designating a new *Candida* species (2).

The type strain of *C. subhashii* was cultured from the peritoneal dialysis effluent of a patient with progressive renal insufficiency (2). The effluent was inoculated onto various standard microbiological media (blood agar, chocolate agar, and MacConkey agar) and yielded fungal colony growth. Colonial features were studied on Sabouraud modified agar and CHROMagar Candida (white colonies) and cornmeal Tween 80 agar (long pseudohyphae and sparse blastoconidia). A negative germ tube test, negative urease test, and API 20C AUX assimilation pattern results did not point to an identity for the isolate. Further testing included conventional fermentation and assimilation tests, as well as additional testing with an extended set of compounds. The DNA sequence of rRNA-encoding genes was determined but did not lead to identification of the isolate as a known species. The combination of data from the various approaches suggested that the isolate belongs to a new *Candida* species, which was named *C. subhashii*. Morphologically, this isolate most closely resembles *Candida tropicalis*, which is a documented cause of fungal peritonitis. Less extensive testing of the isolate, using only morphological criteria, may have identified it as *C. tropicalis*. Identification of the isolate as a new species creates a focal point for evaluation of other *Candida* isolates from cases of fungal peritonitis, some of which are likely to be identified as *C. subhashii*. Once other *C. subhashii* isolates are identified, estimates can be made for the variability that is inherent in this species. Species definition, strain variability, and assessment of its clinical significance are dynamic processes that require reevaluation in light of emerging technologies and increasing knowledge.

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C. albicans provides an outstanding example of the dynamic process of determining the boundaries for a species: "atypical" *C. albicans* isolates have been shown to constitute new species (*C. dubliniensis* [60]), while isolates previously separated into other species have been demonstrated to be *C. albicans* (32). In both examples, initial species assignments were made using nonmolecular diagnostic methods and then refined using DNA sequence information.

The original *C. dubliniensis* isolates were collected from Irish human immunodeficiency virus-infected and AIDS patients and identified as atypical *C. albicans* because they formed germ tubes and chlamydospores, which were previously believed to be associated only with *C. albicans* isolates (59). Attempts to hybridize *C. albicans*-derived DNA fingerprinting probes on Southern blots of *C. dubliniensis* genomic DNA yielded fewer and fainter bands than usually obvious for *C. albicans* DNA (58). This observation led to molecular phylogenetic analyses that suggested that the isolates constituted a new species. Since the recognition of the new species, many methods have been proposed to distinguish between *C. dubliniensis* and its closest relative, *C. albicans* (reviewed in references 10 and 59). Separating *C. dubliniensis* from *C. albicans* isolates has led to improved understanding of the epidemiology, virulence, and antifungal drug resistance of both species.

Jacobsen et al. (32) used multilocus sequence typing (MLST) to investigate the phylogeny of the genome-sequenced isolates of *C. albicans* (SC5314 and WO-1) and isolates of broadly named species (including, for example, *Cryptococcus copellii*, *Mycotoruloides ovalis*, and *Monilia psilosis*) that are now recognized as synonymous with *C. albicans*. MLST is a method that was initially used in bacterial taxonomy (reviewed in reference 41) and has been adapted to address similar questions for fungal species. The consensus method for *C. albicans* includes PCR primers that amplify partial sequences of seven different *C. albicans* genes (*AAT1a*, *ACC1*, *ADP1*, *MPIb*, *SYA1*, *VPS13*, and *ZWF1b* [9]). Single nucleotide polymorphisms (SNPs), identified by DNA sequence analysis of the partial gene fragments, are utilized to determine similarity between isolates. Results can be presented as a dendrogram (Fig. 1) that indicates the clustering of isolates into clades (46). Jacobsen et al. (32) showed that almost all of the isolates tested fit clearly into the established clade structure for the species *C. albicans*. Among the more distantly related isolates were those designated as *Candida africana*, which was described as a species separate from *C. albicans* by Tietz et al. (64). *C. africana* isolates originally were considered atypical *C. albicans* because *C. africana* is germ tube positive. However, *C. africana* isolates grow more slowly than *C. albicans* and do not produce chlamydospores under standardized growth conditions. Also, the strains cannot assimilate *N*-acetylglucosamine, glucosamine, trehalose, or DL-lactate and cannot hydrolyze esculin. Despite designating a new species, the article by Tietz et al. (64) is titled cautiously and inquires if *C. africana* is a new human pathogen or a variant of *C. albicans*. This debate continued in article titles such as the one by Alonso-Vargas et al. (4), which describes *C. africana* isolates as "probable atypical strains of *C. albicans*." Despite the ability to distinguish *C. africana* isolates from *C. albicans* based on morphological and physiological assays, DNA sequence data show near identity between sequences of the genes encoding the 26S rRNA from *C. africana* and *C. albicans* (4). While reporting the first Italian isolate of *C. africana*, Romeo and Criseo (49) proposed *Candida albicans* var. *africana* as a name for the strains. *C. albicans* MLST primers amplified all seven gene fragments from *C. africana* isolates and placed them in clade 13, which was the cluster least similar to the rest of the analyzed isolates (Fig. 1; 32, 46). Based on these data, *C. africana* was concluded to be synonymous with *C. albicans*.

The examples discussed so far showed how a mixture of morphological, physiological, and molecular methods was used to define a new *Candida* species, *C. subhashii*. The examples also showed how molecular analysis can confirm that atypical isolates are really a new species, or that despite obvious morphological and physiological differences between isolates, molecular analysis cannot separate them into a new species. A different look at the issue of microbial speciation is found in the example of *Candida parapsilosis*. Multilocus enzyme electrophoresis and many different molecular analyses showed that isolates identified as *C. parapsilosis* could be separated into three groups, which were called I, II, and III (reviewed in reference 62). Tavanti et al. (62) attempted to devise an MLST scheme for *C. parapsilosis* and, again, noted three groups of isolates. Reclassification of the groups as separate species was proposed despite the lack of detection of phenotypic differences between the isolates for sugar assimilation profiles, appearance on CHROMagar Candida, growth temperature parameters, biofilm formation, or antifungal susceptibility results. To indicate that there are few differences between the species, group I was retained as *C. parapsilosis*, while groups II and III were given related names (*Candida orthopsilosis* and *Candida metapsilosis*, respectively). Subsequent work with larger strain collections suggested that there are differences in antifungal susceptibility measurements for the different species (23, 37, 65).

The dynamic process of assigning isolates to species is not unique to examples within the genus *Candida* or to eukaryotic microbes. The concept and meaning of the term species in microbial systematics are being questioned and reconsidered in light of the abundance of emerging genomic information (1). While the overall debate about microbial species designations continues, the examples provided here show some of the criteria that have been used recently to make species designations within the genus *Candida*. A discussion of variability among isolates within the same species is dependent on these taxonomic decisions.

DETECTION AND TYPES OF VARIABILITY AMONG ISOLATES WITHIN A *CANDIDA* SPECIES

One common reason to study the genetic relatedness among isolates is to determine the population structure for a species. It is accepted that *C. albicans* has a mostly clonal population structure (24), although mating between strains occurs to form tetraploids that experience an unpredictable loss of chromosomes (reviewed in reference 6). Methods such as DNA fingerprinting provided the initial insights into the genetic groups (clades) of *C. albicans* isolates. For this method, Southern blots of restriction fragments of genomic DNA were hybridized with complex DNA probes, the most common called Ca3 (reviewed in reference 56). More recently, DNA sequence-based methods have emerged for describing population structure, with MLST most commonly used (discussed above). For *C. albicans*, results from DNA fingerprinting are in agreement with those from MLST (46). Figure 1 shows the phylogeny derived from MLST analysis of 1,391 *C. albicans* isolates. Both DNA

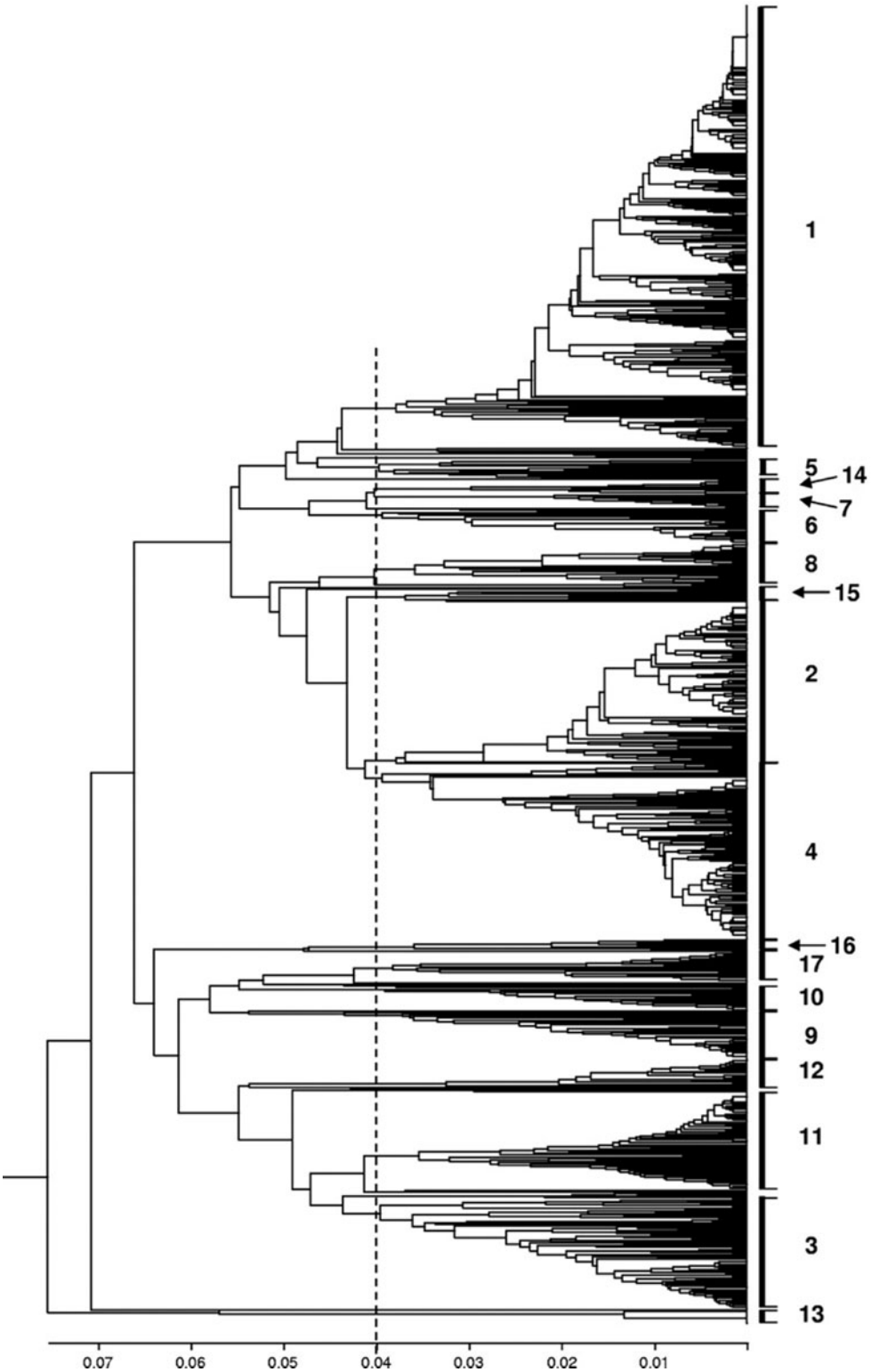

FIGURE 1 Dendrogram constructed using the unweighted-pair group method with arithmetic mean for 1,391 *C. albicans* isolates typed by MLST (46). The scale at the bottom shows *P* distances, with the vertical dashed line at the *P* value of 0.04 used to designate clades. Clade numbers are shown on the right, with vertical bars to indicate their relative sizes. *C. africana* isolates are placed into clade 13, which is the cluster that is least similar to the others (32, 46). Reprinted from *Eukaryotic Cell* (46) with permission of the publisher. 10.1128/9781555817176.ch7f1

fingerprinting and MLST have been applied to the study of *C. dubliniensis* population structure; the two methods produced similar conclusions (42). Fingerprinting used the species-specific probe Cd25 and the MLST scheme included eight loci. Analysis revealed a population structure of three major clades that appears much less divergent than the population structure observed for *C. albicans*. MLST methods have also been developed for *Candida glabrata* (17), *Candida tropicalis* (63), and *Candida krusei* (34).

Studies of *Candida* genetic variability may also be conducted from the perspective of assessing genomic rearrangements. The nature of genomic rearrangements in the diploid *C. albicans* was understood more clearly from construction of a physical map of its eight pairs of chromosomes (called 1 to 7 and R [13]). For this work, pulsed-field gel electrophoresis-separated chromosomes and restriction enzyme-digested genomic DNA were Southern blotted with probes corresponding to various genomic sequences. The presence and importance of the *C. albicans* major repeat sequence (MRS) were understood more fully as this work progressed. The MRS is an intermediate repeat element that is unrelated to retrotransposons (reviewed in reference 12). At this time, it is understood that the MRS is associated with chromosome translocations and chromosome-length polymorphisms (12). The MRS comprises approximately 1 to 2% of the *C. albicans* genome and occurs nine times in the haploid genome. DNA fingerprinting probe sequences useful in phylogenetic and epidemiological studies (such as Ca3; see above) are derived from the MRS. The MRS is also present in the closely related *C. dubliniensis* (12).

In addition to experiencing genomic rearrangements, isolates of the diploid *C. albicans* often are aneuploid (either gain or loss of a whole or partial chromosome). Several methods are available to detect aneuploidy. One example is electrophoretic separation of chromosomes or chromosome fragments, often accompanied by Southern blotting (reviewed in reference 50). Comparative genome hybridization (CGH) was developed more recently as a method for aneuploidy detection (7). In this method, fluorescently labeled digested genomic DNA from the strain of interest is hybridized to a microarray and relative copy numbers are calculated for the various array probes. Identifying the location of the nondiploid probes with respect to the *C. albicans* genome sequence shows which chromosomes or chromosomal fragments are gained or lost between isolates. Aneuploidy can also be inferred from analysis of whole-genome transcript profiling data (5, 7). Aneuploidy appears to be a response of *C. albicans* to stressful conditions, such as growth in conditions of nutritional stress (reviewed in reference 50), or exposure to antifungal drugs (see below). *C. albicans* strains can be aneuploid naturally (on initial isolation from a clinical specimen; reviewed in reference 50) or can become aneuploid as the result of laboratory manipulations such as mutant construction (3, 5, 7, 52). The high frequency of aneuploid isolates constructed for use in laboratory experiments has led to the suggestion that new constructs be evaluated for this characteristic. Arbour et al. (5) described a multiplex PCR aneuploidy detection assay using two multiplex primer sets, each amplifying a sequence from the left arm (set A) or right arm (set B) of each of the eight *C. albicans* chromosomes. Altered band intensity within each primer set is used to signal aneuploidy. Ensuring a lack of aneuploidy in mutant constructs intended for phenotypic analysis would increase the accuracy and meaning of data from microarray analysis, for example, where copy number is important for deciding which genes are up- and downregulated in relation to a control strain.

Events such as mutation and mitotic recombination also can contribute to variability among *C. albicans* strains. The diploid genome sequence of *C. albicans* strain SC5314 showed more than 56,000 SNPs (approximately 1 per 237 bases [35]). Alterations in SNPs are evidence that events such as mutation and mitotic recombination have occurred. These processes can lead to loss of heterozygosity (LOH), which has been demonstrated to affect such important phenotypes as antifungal drug resistance (see below). Several methods have been developed to utilize SNPs for variability detection. Forche et al. (18) constructed a genome-wide SNP map based on the *C. albicans* strain SC5314 genome sequence. The SNP map was used to construct a SNP microarray for chromosomes 5, 6, and 7 that could be used to identify mitotic recombination events that occurred during infection in mice (see below and reference 21). Forche et al. (22) also developed a non-microarray-based method to detect LOH at individual loci, as well as over larger regions such as chromosome arms or whole chromosomes. This method uses SNP-restriction fragment length polymorphism (RFLP) markers. Four markers were defined for each of the eight chromosomes; each SNP-RFLP marker has a polymorphic restriction enzyme site that produces restriction fragments that are identifiable by size. The SNP-RFLP method is a more economical alternative to array-based methods and provides a rapid screen to detect genetic changes in strains. In another study, the combination of CGH and SNP microarrays was used to construct a haplotype map for *C. albicans* SC5314 (36). With this approach, the arrangement of SNPs on homologous chromosomes (linkage in *cis* or *trans*) can be determined using strains that are homozygous or trisomic for a given chromosome.

In some studies, variability between *C. albicans* isolates is detected during studies of a specific gene, rather than from analysis of larger genomic regions or of loci selected due to the presence of polymorphisms. One example can be found in the eight genes of the ALS (agglutinin-like sequence) family that encode large cell surface glycoproteins that are most commonly discussed with respect to adhesive function (reviewed in references 26 and 27). The most simplistic way to view ALS gene structure is a central domain consisting entirely of tandem copies of a highly conserved 108-bp motif, flanked by a 5′ domain (which has adhesive capacity) and a 3′ domain (encoding a serine/threonine-rich, heavily glycosylated portion of each Als protein). Alleles of the same ALS gene frequently encode a different number of copies of the repeated unit in the central domain, providing extensive variability to ALS alleles. For some ALS genes, other sequence differences are common, including a wide range of sequence variations in the 5′ domain of *ALS5* (28, 71) and extensive sequence variability in the 3′ domain of *ALS7* (69) and 5′ and 3′ domains of *ALS9* (70, 72). Extensive sequence variation is also noted in the fragments of the seven *C. albicans* genes that are used in the MLST method. At present, there are over 1,500 diploid sequence types (DSTs) defined by MLST, with at least 100, and in some cases closer to 200, allelic sequence variants defined for the different genes (http://calbicans.mlst.net). Odds (45) noted the possibility that this sequence information could lead to chip-based strain typing systems in the future.

Studies of variability among *C. albicans* isolates have demonstrated that the species has an extensive capacity for genetic change. Methodologies used for studying *C. albicans*

are being applied to other pathogenic *Candida* species. A recent report described CGH analysis of *C. glabrata* from French hospitals (43). Among the observations were rare instances of reciprocal chromosomal translocations and recombination within tandem arrays of repeated genes. As methods are applied to a greater number of *Candida* species, it will become more apparent if the genomic plasticity observed for *C. albicans* is a common characteristic or limited to only a few species.

CLINICAL SIGNIFICANCE OF *CANDIDA* STRAIN VARIATION

Since pathogenic *Candida* species are examined with the intent of understanding host-*Candida* interaction and disease interventions, all analyses of pathogenic *Candida* species have clinical significance. Most isolates that are studied are derived from clinical specimens; their presence at the sampled site indicates at least a transient or commensal association with the host, or that the isolate is the cause of a disease process. Studies of the clinical significance of *C. albicans* genetic variability have been conducted from different perspectives, including inquiries into the associations between *C. albicans* clades and variables of clinical significance and investigations into the degree of strain variation that is found among clinical isolates from both commensal and disease states, as well as the changes that occur during exposure to antifungal drugs. A selection of these topics is presented here, with an emphasis on *C. albicans*.

Various *C. albicans* strain typing studies have found a significant association between geographical location and clade distribution of isolates (46, 56). Isolates from the most common clades can be found in most geographical locations, but certain clades are enriched in certain parts of the world. For example, clade 4 isolates are particularly enriched in South Africa, while clade 2 isolates are common in the United Kingdom but relatively rare in the southwestern United States. Geographically enriched clades are also observed in MLST data for *C. glabrata* (17). Enrichment of *Candida* clades is observed, despite the frequency of human travel that provides homogenization among isolates. One study examined whether the local wildlife population was responsible for maintaining a reservoir of *C. albicans* isolates specific to a defined geographic area in the midwestern United States (66). In this work, *C. albicans* isolates and travel history were collected from humans and isolates were also collected from nonmigratory wildlife. The work was expanded to include collection of *C. albicans* isolates from domestic animals. Results showed that there is a significant difference in the clade distribution of isolates from humans and wildlife, demonstrating population isolation between the groups. Jacobsen et al. (33) observed a similar result from analysis of a wide range of *C. albicans* isolates from animals. Wrobel et al. (66) found that isolates from companion animals were rare and, when detected, represented the most common human DSTs. The results suggested that nontransient humans most likely preserve the connection between geography and *C. albicans* clades. Results also suggested that *C. albicans* has only a temporary association with domestic animals such as dogs and cats and that transfer of *C. albicans* is more likely to occur from humans to animals than from animals to humans.

The results of *C. albicans* strain typing can be used to address the question of whether one or a few particular strain types account for the majority of clinical cases. Analysis of MLST data by Odds et al. (46) showed no significant relationship between strain type and disseminated candidiasis but demonstrated the tendency for clade 1 isolates to be recovered as commensals or from superficial disease. Schmid et al. (51) described a general-purpose genotype for *C. albicans* that was more likely to be associated with cases of human disease. Isolates displaying the general-purpose genotype correspond to those in clade 1 (46).

Strain typing methods have shown that the *C. albicans* isolate responsible for disseminated infection in humans is usually derived from the patient, rather than from external sources (reviewed in reference 45). However, examples of outright *C. albicans* strain replacement in a human patient are also documented. The *C. albicans* literature is rich in examples of studies that show that the same or a closely related strain is present on a given patient at multiple body locations, or over a period of time from collection of sequential isolates (reviewed in reference 45). More recent studies have characterized genetic variability between isolates from the same anatomical site on the SNP level and addressed the question of how much genomic change occurs for *C. albicans* strains in a host, in both commensal and disease states. Examples of genetic changes in isolates collected from patients treated with antifungal drugs are numerous and demonstrate the tremendous plasticity of the *C. albicans* genome in the presence of antifungal drug exposure (see below). Bougnoux et al. (8) focused on commensal isolates collected from the gastrointestinal tracts of individuals and individuals within the same family. The isolates were characterized by MLST, which allowed for detection of SNPs in the seven gene fragments that constitute the consensus MLST method for *C. albicans*. Evidence of LOH was shown for loci in isolates collected from an individual or between isolates for individuals in the same family. Wrobel et al. (66) observed LOH in isolates from the same individual in a collection of wildlife isolates, suggesting that similar processes function in animals and humans. Study of the human gastrointestinal commensal isolates was expanded to examine the molecular basis for the LOH events (15). Results showed that sequence changes were due to large LOH events such as allelic recombination, break-induced replication, or chromosome loss and duplication of the remaining chromosome. Commensal isolates also showed more localized changes due to gene conversion, as well as karyotypic changes such as chromosome length polymorphism and variations in chromosome copy number. These observations suggest extensive plasticity of the *C. albicans* genome in the commensal state.

Assessments of genetic change within a host have also been accomplished using animal disease models. Forche et al. (20) used *GAL1* as a selectable marker to study *C. albicans* mitotic recombination and gene conversion in a murine model of disseminated candidiasis. In this model, *C. albicans* cells are injected into the lateral tail vein of the mouse. Infection is allowed to progress and *C. albicans* colonies are recovered following excision and homogenization of the kidneys and plating the homogenate on agar medium. Forche et al. (20) created strains heterozygous at the *GAL1* locus and measured the ability of strains to grow on 2-deoxygalactose following isolation from the mouse kidney. The frequency of mitotic recombinants was estimated in the range of 10^{-2} to 10^{-4}. The 2-deoxygalactose-resistant strains were evaluated further using a SNP microarray focused on chromosomes 5, 6, and 7 (21). LOH events that were

observed were likely due to gene conversion, since chromosomal regions that retained heterozygosity separated the events. Strains were karyotyped using pulsed-field gel electrophoresis and evaluated for growth rate, colony morphology, and germ tube formation in serum. Individual strains were found to exhibit LOH and all, or any combination, of these properties. Further work with the *gal1/GAL1* *C. albicans* strains compared genetic and phenotypic changes in the strains as they were passaged in a mouse model compared to passage in a culture flask (19). The authors concluded that in vivo passage resulted in slower population growth and higher rates of genomic and phenotypic change than in the in vitro culture.

The effect of genomic changes on virulence has been assessed using the murine model of disseminated candidiasis. Chen et al. (11) noted the presence of three copies of chromosome 1 in a common *C. albicans* laboratory strain and demonstrated that strains with chromosome 1 trisomy were avirulent in the mouse model. In another study, Wu et al. (67) examined the idea that heterozygosity of genes on *C. albicans* chromosome 5, on which the mating-type locus (MTL [29]) is located, regulates virulence. In the diploid *C. albicans*, the MTL is heterozygous in most isolates, with MAT**a** on one chromosome 5 homolog and MATα on the other (46). Mating can occur between **a/a** and α/α cells, requiring homozygosity at the MTL (30, 40). MTL homozygosity can result from the loss of one chromosome 5 homolog and duplication of the other (uniparental disomy [68]). Strains that are homozygous at the MAT locus are less virulent than MAT heterozygous strains (31, 38). Wu et al. (67) explored the role that heterozygosity plays across chromosome 5 as a whole and found that LOH at the MAT locus results in a small decrease in virulence in the mouse model, while LOH at other chromosome 5 loci results in a much greater reduction in virulence. These results suggest a model where MTL homozygosis in natural isolates occurs by multiple crossover events on chromosome 5, rather than by uniparental disomy.

Variability in genes encoding virulence factors has been observed for *C. albicans*. For example, the alleles of *SAP2*, which encodes one of a family of secreted aspartyl proteinases in *C. albicans* strain SC5314, are differentially regulated in vitro and in vivo (57). In the murine model of disseminated disease, the *SAP2-2* allele is induced earlier than the *SAP2-1* allele. The difference in expression is due to a variable number of copies of two pentameric nucleotide repeat sequences in the promoter of each allele. As discussed above, alleles of ALS genes frequently vary in the number of copies of the tandemly repeated sequence found in the central domain of the coding region. Studies of *ALS3*, *ALS5*, and *ALS6* examined whether tandem repeat copy number varied significantly by clade (47, 71). For *ALS3*, the prevalent alleles and the allele distribution differed between clades, although clades were similar with respect to the mean repeat copy number for *ALS3* alleles. *C. albicans* isolates show a strong tendency toward heterozygosity, with one *ALS3* allele encoding 12 or more copies of the repeat unit and the other allele encoding fewer than 12 copies. The 12-copy *ALS3* allele in strain SC5314 contributed obvious adhesive function to *C. albicans* when assayed for binding to cultured epithelial or endothelial cell monolayers, while the adhesive contribution from the smaller (9-repeat copies) allele was measurable but minor. Analysis of tandem-repeat copy number in *ALS5* and *ALS6* alleles showed similarity to results for *ALS3*. For both *ALS5* and *ALS6*, prevalent alleles and allele distributions varied among the clades (71). Isolates in which *ALS5* is deleted either on one or both homologs of chromosome 6 were identified. Each clade had isolates in which one *ALS5* copy was missing (hemizygous strains), but clade 2, where the percentage of hemizygous strains was greatest, did not have any strains where both *ALS5* copies were deleted. Clade SA (equivalent to clade 4 in the MLST phylogeny [Fig. 1]) had the greatest percentage of *ALS5*-deleted strains. The phenotypic effect of homozygous *ALS5* deletion among wild-type clinical isolates is not known. Sequence differences in the 5′ domain of *ALS9* lead to proteins with differential adhesive function (70). In an assay to measure *C. albicans* adhesion to cultured vascular endothelial cell monolayers, Als9-2 contributed adhesive function, while Als9-1 did not. Analysis of *ALS9* allelic frequency in a collection of geographically diverse clinical isolates showed a distinct preference for *ALS9-2* allelic sequences.

MacCallum et al. (39) addressed whether there are phenotypic differences between strains from different clades. Multiple strains from the four largest clades (1, 2, 3, and 4; 43 strains total) were assessed for virulence in mice, growth rate in vitro, biofilm formation on polystyrene, acid phosphatase specific activity, Alcian blue binding, growth in 2 M NaCl, and adhesion to human buccal epithelial cells and catheter plastic. There was no significant association between clade and virulence in mice. Examination of the other traits showed a significantly lower acid phosphatase specific activity for clade 2 strains and the tendency for more clade 1 strains to grow in 2 M NaCl. Expression profiling using two different growth conditions suggested that strains from clade 3 had the most distinctive interclade expression differences. The number of copies of repeated sequences in the coding regions of some ALS genes and also in genes from the HYR family (encoding predicted cell surface glycoproteins) was significant by clade. As described above, previous studies of ALS genes from a larger strain collection analyzed clade differences in repeat copy numbers and showed significant differences between clades for *ALS3*, *ALS5*, and *ALS6* (47, 71). The analysis of ALS repeat copy number by MacCallum et al. (39) is problematic because some primer pairs were not specific for the ALS gene studied (*ALS2* and *ALS4*, for example), while another primer pair (*ALS9*) included additional sequences 3′ of the central tandem repeat domain that vary between alleles. Regardless of these issues, this work increases the list of properties that differ between strains from the largest *C. albicans* clades.

C. albicans strain variation is associated with antifungal drug resistance, a problem of obvious clinical significance. For *C. albicans*, resistance to certain antifungal drugs is associated with specific clades. Pujol et al. (48) showed that flucytosine resistance is found only in isolates from clade 1. Further work demonstrated that the resistance is due to a single nucleotide change in the *FUR1* gene, which encodes uracil phosphoribosyltransferase (16). Analysis of a larger strain collection showed flucytosine-resistant isolates that belonged to clades other than clade 1 (61). These strains, however, did not share the *FUR1* mutation observed for the clade 1 isolates, nor did they show mutations in other genes that might explain flucytosine resistance. Strains in clade 1 were more likely to exhibit terbinafine resistance, although isolates that were cross-resistant to flucytosine were rare (44). Similar to the *FUR1* data, another study provided evidence of a small number of nucleotide changes required for a notable change in antifungal drug resistance. Holmes et al. (25) showed the association between two adjacent SNPs in

CDR2 (which encodes a plasma membrane drug efflux pump) and azole susceptibility.

Large chromosomal changes can be involved in antifungal drug resistance mechanisms. Selmecki et al. (53) used CGH to analyze gene copy number at all loci in a collection of azole-resistant and azole-sensitive strains. Aneuploidy (trisomy of chromosome 5, as well as segmental aneuploidy) was detected far more commonly in the resistant isolates. Distinctive gene copy number differences were similar among the resistant isolates, leading to the discovery of an isochromosome (two identical chromosome arms separated by a centromere) that consisted of two chromosome 5L arms. This chromosomal fragment includes *ERG11*, which encodes the target for azole drugs, and *TAC1*, which encodes a transcription factor that regulates drug efflux pump-encoding genes (*CDR1* and *CDR2*, for example). Involvement of these genes in the azole resistance mechanism was demonstrated by analysis of a set of *C. albicans* isolates collected over time from a bone marrow transplant patient who was treated with antifungal drugs (54). In this strain series, increasing the copy number of *ERG11* and *TAC1* made additive, but independent, contributions to azole drug resistance. Follow-up experiments using in vitro populations of cells treated with fluconazole showed the appearance of isochromosome 5L shortly after initiation of drug exposure (55). Aneuploidies of other chromosomes, as well as attachment of isochromosome 5L to other chromosomes, were observed in cells that had the highest fluconazole resistance. Development of aneuploidy appears to increase the fitness of *C. albicans* cells that are exposed to fluconazole.

The study by Coste et al. (14) examined *C. albicans* genetic variability ranging from large chromosomal changes to point mutations in the context of azole resistance. The work utilized several sets of sequential isolates collected either from patients or from an extended period of in vitro passage. Known mutations in *ERG11* were found in isolates with azole resistance, along with many newly identified *TAC1* alleles, several of which were hyperactive due to changes such as gain-of-function point mutations. LOH events were demonstrated by SNP analysis and were responsible for homozygosity of the alleles that conferred azole resistance. Whole and segmental aneuploidy of chromosome 5 was observed, as well as formation of isochromosome 5L in some isolates. The observed genetic changes were detected in various combinations and revealed sophisticated genotypes for azole-resistant isolates. The work demonstrates the impressive display of genetic variability that *C. albicans* can develop when challenged with exposure to antifungal drugs.

SUMMARY AND CONCLUSIONS

Species assignment is based increasingly on molecular criteria. These methods may result in the same species assignment for isolates with obvious phenotypic differences or may assign two isolates to different species despite a lack of detectable phenotypic differences. A given isolate of *C. albicans* can undergo an impressive range of genetic changes at the level of point mutation to alterations in whole chromosomes. These may occur during laboratory manipulation of the strain or in vivo as a commensal or in the context of disease and exposure to antifungal drugs. Genetic changes have demonstrable phenotypic effects in traits of clinical relevance. As analysis of pathogenic *Candida* species expands from its *C. albicans*-centered focus, we will understand whether these themes are also observed for other *Candida* species.

REFERENCES

1. **Achtman, M., and M. Wagner.** 2008. Microbial diversity and the genetic nature of microbial species. *Nat. Rev. Microbiol.* **6:**431–440.
2. **Adam, H., M. Groenewald, S. Mohan, S. Richardson, U. Bunn, C. F. C. Gibas, S. Poutman, and L. Sigler.** 2009. Identification of new species, *Candida subhashii*, as a cause of peritonitis. *Med. Mycol.* **47:**305–311.
3. **Ahmad, A., M. Anaul Kabir, A. Kravets, E. Andaluz, G. Larriba, and E. Rustchenko.** 2008. Chromosome instability and unusual features of some widely used strains of *Candida albicans*. *Yeast* **25:**433–448.
4. **Alonso-Vargas, R., L. Elorduy, E. Eraso, J. F. Canos, J. Guarros, J. Ponton, and G. Quindos.** 2008. Isolation of *Candida africana*, probable atypical strains of *Candida albicans*, from a patient with vaginitis. *Med. Mycol.* **46:** 167–170.
5. **Arbour, M., E. Epp, H. Hogues, A. Sellam, C. Lacroix, J. Rauceo, A. Mitchell, M. Whiteway, and A. Nantel.** 2009. Widespread occurrence of chromosomal aneuploidy following the routine production of *Candida albicans* mutants. *FEMS Yeast Res.* **9:**1070–1077.
6. **Bennett, R. J., and A. D. Johnson.** 2005. Mating in *Candida albicans* and the search for a sexual cycle. *Annu. Rev. Microbiol.* **59:**233–255.
7. **Bouchonville, K., A. Forche, K. E. S. Tang, A. Selmecki, and J. Berman.** 2009. Aneuploid chromosomes are highly unstable during DNA transformation of *Candida albicans*. *Eukayot. Cell* **8:**1554–1566.
8. **Bougnoux, M.-E., D. Diogo, N. François, B. Sendid, S. Veirmeire, J. F. Colombel, C. Bouchier, H. Van Kruiningen, C. d'Enfert, and D. Poulain.** 2006. Multilocus sequence typing reveals intrafamilial transmission and microevolutions of *Candida albicans* isolates from the human digestive tract. *J. Clin. Microbiol.* 44:1810–1820.
9. **Bougnoux, M.-E., A. Tavanti, C. Bouchier, N. A. R. Gow, A. Magnier, A. D. Davidson, M. C. J. Maiden, C. d'Enfert, and F. C. Odds.** 2003. Collaborative consensus for optimized multilocus sequence typing of *Candida albicans*. *J. Clin. Microbiol.* **41:**5265–5266.
10. **Campanha, N. H., K. H. Neppelenbroek, D. M. Spolidorio, L. C. Spolidorio, and A. C. Pavarina.** 2005. Phenotypic methods and commercial systems for the discrimination between *C. albicans* and *C. dubliniensis*. *Oral Dis.* **11:**392–398.
11. **Chen, X., B. B. Magee, D. Dawson, P. T. Magee, and C. A. Kumamoto.** 2004. Chromosome 1 trisomy compromises the virulence of *Candida albicans*. *Mol. Microbiol.* **51:** 551–565.
12. **Chibana, H., and P. T. Magee.** 2009. The enigma of the major repeat sequence of *Candida albicans*. *Future Microbiol.* **4:**171–179.
13. **Chu, W. S., B. B. Magee, and P. T. Magee.** 1993. Construction of an *Sfi*I macrorestriction map of the *Candida albicans* genome. *J. Bacteriol.* **175:**6637–6651.
14. **Coste, A., A. Selmecki, A. Forche, D. Diogo, M.-E. Bougnoux, C. d'Enfert, J. Berman, and D. Sanglard.** 2007. Genotypic evolution of azole resistance mechanisms in sequential *Candida albicans* isolates. *Eukaryot. Cell* **6:**1889–1904.
15. **Diogo, D., C. Bouchier, C. d'Enfert, and M.-E. Bougnoux.** 2009. Loss of heterozygosity in commensal isolates of the asexual diploid yeast *Candida albicans*. *Fungal Genet. Biol.* **46:**159–168.
16. **Dodgson, A. R., K. J. Dodgson, C. Pujol, M. A. Pfaller, and D. R. Soll.** 2004. Clade-specific flucytosine resistance is due to a single nucleotide change in the *FUR1* gene of *Candida albicans*. *Antimicrob. Agents Chemother.* **48:** 2223–2227.

17. **Dodgson, A. R., C. Pujol, D. W. Denning, D. R. Soll, and A. J. Fox.** 2003. Multilocus sequence typing of *Candida glabrata* reveals geographically enriched clades. *J. Clin. Microbiol.* **41:**5709–5717.
18. **Forche, A., P. T. Magee, B. B. Magee, and G. May.** 2004. Genome-wide single-nucleotide polymorphism map for *Candida albicans*. *Eukaryot. Cell* **3:**705–714.
19. **Forche, A., P. T. Magee, A. Selmecki, J. Berman, and G. May.** 2009. Evolution in *Candida albicans* populations during a single passage through a mouse host. *Genetics* **182:**799–811.
20. **Forche, A., G. May, J. Beckerman, S. Kauffman, J. Becker, and P. T. Magee.** 2003. A system for studying genetic changes in *Candida albicans* during infection. *Fungal Genet. Biol.* **39:**38–50.
21. **Forche, A., G. May, and P. T. Magee.** 2005. Demonstration of loss of heterozygosity by single-nucleotide polymorphism microarray analysis and alterations in strain morphology in *Candida albicans* strains during infection. *Eukaryot. Cell* **4:**156–165.
22. **Forche, A., M. Steinbach, and J. Berman.** 2009. Efficient and rapid identification of *Candida albicans* allelic status using SNP-RFLP. *FEMS Yeast Res.* **9:**1061–1069.
23. **Gonçalves, S. S., C. S. Amorim, M. Nucci, A. C. B. Padovan, M. R. S. Briones, A. S. A. Melo, and A. L. Colombo.** 2010. Prevalence rates and antifungal susceptibility profiles of the *Candida parapsilosis* species complex: results from a nationwide surveillance of candidaemia in Brazil. *Clin. Microbiol. Infect.* **16:**885–887.
24. **Gräser, Y., M. Volovsek, J. Arrington, G. Schonian, W. Presber, T. G. Mitchell, and R. Vilgalys.** 1996. Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. *Proc. Natl. Acad. Sci. USA* **93:** 12473–12477.
25. **Holmes, A. R., S. Tsao, S.-W. Ong, E. Lamping, K. Niimi, B. C. Monk, M. Niimi, A. Kaneko, B. R. Holland, J. Schmid, and R. D. Cannon.** 2006. Heterozygosity and functional allelic variation in the *Candida albicans* efflux pump genes *CDR1* and *CDR2*. *Mol. Microbiol.* **62:**170–186.
26. **Hoyer, L. L.** 2001. The ALS gene family of *Candida albicans*. *Trends Microbiol.* **9:**176–180.
27. **Hoyer, L. L., C. B. Green, S.-H. Oh, and X. Zhao.** 2008. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family—a sticky pursuit. *Med. Mycol.* **46:**1–15.
28. **Hoyer, L. L., and J. E. Hecht.** 2001. The *ALS5* gene of *Candida albicans* and analysis of the Als5p N-terminal domain. *Yeast* **18:**49–60.
29. **Hull, C. M., and A. D. Johnson.** 1999. Identification of a mating type-like locus in the asexual pathogenic yeast *Candida albicans*. *Science* **285:**1271–1275.
30. **Hull, C. M., R. M. Raisner, and A. D. Johnson.** 2000. Evidence for mating of the 'asexual' yeast *Candida albicans* in a mammalian host. *Science* **289:**307–310.
31. **Ibrahim, A. S., B. B. Magee, D. C. Sheppard, M. Yang, S. Kauffman, J. Becker, J. E. Edwards, Jr., and P. T. Magee.** 2005. Effects of ploidy and mating type on virulence of *Candida albicans*. *Infect. Immun.* **73:**7366–7374.
32. **Jacobsen, M. D., T. Boekhout, and F. C. Odds.** 2008. Multilocus sequence typing confirms synonymy but highlights differences between *Candida albicans* and *Candida stellatoidea*. *FEMS Yeast Res.* **8:**764–770.
33. **Jacobsen, M. D., M.-E. Bougnoux, C. d'Enfert, and F. C. Odds.** 2008. Multilocus sequence typing of *Candida albicans* isolates from animals. *Res. Microbiol.* **159:**436–440.
34. **Jacobsen, M. D., N. A. Gow, M. C. Maiden, D. J. Shaw, and F. C. Odds.** 2007. Strain typing and determination of population structure of *Candida krusei* by multilocus sequence typing. *J. Clin. Microbiol.* **45:**317–323.
35. **Jones, T., N. A. Federspiel, H. Chibana, J. Dungan, S. Kalman, B. B. Magee, G. Newport, Y. R. Thorstenson, N. Agabian, P. T. Magee, R. W. Davis, and S. Scherer.** 2004. The diploid genome sequence of *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **101:**7329–7334.
36. **Legrand, M., A. Forche, A. Selmecki, C. Chan, D. T. Kirkpatrick, and J. Berman.** 2008. Haplotype mapping of a diploid non-meiotic organism using existing and induced aneuploidies. *PLoS Genet.* **4:**18–28.
37. **Lockhart, S. R., S. A. Messer, M. A. Pfaller, and D. J. Diekema.** 2008. Geographic distribution and antifungal susceptibility of the newly described species *Candida orthopsilosis* and *Candida metapsilosis* in comparison to the closely related species *Candida parapsilosis*. *J. Clin. Microbiol.* **46:**2659–2664.
38. **Lockhart, S. R., W. Wu, J. B. Radke, R. Zhao, and D. R. Soll.** 2005. Increased virulence and competitive advantage of a/alpha over a/a or alpha/alpha offspring conserves the mating system of *Candida albicans*. *Genetics* **169:**1883–1890.
39. **MacCallum, D. M., L. Castillo, K. Nather, C. A. Munro, A. J. P. Brown, N. A. Gow, and F. C. Odds.** 2009. Property differences among the four major *Candida albicans* strain clades. *Eukaryot. Cell* **8:**373–387.
40. **Magee, B. B., and P. T. Magee.** 2000. Induction of mating in *Candida albicans* by construction of MTLa and MTLalpha strains. *Science* **289:**310–313.
41. **Maiden, M. C.** 2006. Multilocus sequence typing of bacteria. *Annu. Rev. Microbiol.* **60:**561–588.
42. **McManus, B. A., D. C. Coleman, G. Moran, E. Pinjon, D. Diogo, M.-E. Bougnoux, S. Borecká-Melkusova, H. Bujdaková, P. Murphy, C. d'Enfert, and D. J. Sullivan.** 2008. Multilocus sequence typing reveals that the population structure of *Candida dubliniensis* is significantly less divergent than that of *Candida albicans*. *J. Clin. Microbiol.* **46:**652–664.
43. **Muller, H., A. Thierry, J. Y. Coppee, C. Hennequin, O. Sismeiro, E. Talla, B. Dujon, and C. Fairhead.** 2009. Genomic polymorphism in the population of *Candida glabrata*: gene copy-number variation and chromosomal translocations. *Fungal Genet. Biol.* **46:**264–276.
44. **Odds, F. C.** 2009. In *Candida albicans*, resistance to flucytosine and terbinafine is linked to MAT locus homozygosity and multilocus sequence typing clade 1. *FEMS Yeast Res.* **9:**1091–1101.
45. **Odds, F. C.** 2010. Molecular phylogenetics and epidemiology of *Candida albicans*. *Future Microbiol.* **5:**67–79.
46. **Odds, F. C., M. E. Bougnoux, D. J. Shaw, J. M. Bain, A. D. Davidson, D. Diogo, M. D. Jacobsen, M. Lecomte, S. Y. Li, A. Tavnati, M. C. Maiden, N. A. Gow, and C. d'Enfert.** 2007. Molecular phylogenetics of *Candida albicans*. *Eukaryot. Cell* **6:**1041–1052.
47. **Oh, S.-H., G. Cheng, J. A. Nuessen, R. Jajko, K. M. Yeater, X. Zhao, C. Pujol, D. R. Soll, and L. L. Hoyer.** 2005. Functional specificity of *Candida albicans* Als3p proteins and clade specificity of *ALS3* alleles discriminated by the number of copies of the tandem repeat sequence in the central domain. *Microbiology* **151:**673–681.
48. **Pujol, C., M. A. Pfaller, and D. R. Soll.** 2004. Flucytosine resistance is restricted to a single genetic clade of *Candida albicans*. *Antimicrob. Agents Chemother.* **48:**262–266.
49. **Romeo, O., and G. Criseo.** 2008. Morphological, biochemical and molecular characterization of the first Italian *Candida africana* isolate. *Mycoses* **52:**454–457.
50. **Rustchenko, E.** 2007. Chromosome instability in *Candida albicans*. *FEMS Yeast Res.* **7:**2–11.
51. **Schmid, J., S. Herd, P. R. Hunter, R. D. Cannon, M. S. Yasin, S. Samad, M. Carr, D. Parr, W. McKinney,**

M. Schousboe, B. Harris, R. Ikram, M. Harris, A. Restrepo, G. Hoyos, and K. P. Singh. 1999. Evidence for a general-purpose genotype in *Candida albicans*, highly prevalent in multiple geographical regions, patient types and types of infection. *Microbiology* **145:**2405–2413.

52. **Selmecki, A., S. Bergmann, and J. Berman.** 2005. Comparative genome hybridization reveals widespread aneuploidy in *Candida albicans* laboratory strains. *Mol. Microbiol.* **55:**1553–1565.
53. **Selmecki, A., A. Forche, and J. Berman.** 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science* **313:**367–370.
54. **Selmecki, A., M. Gerami-Nejad, C. Paulson, A. Forche, and J. Berman.** 2008. An isochromosome confers drug resistance in vivo by amplification of two genes, *ERG11* and *TAC1*. *Mol. Microbiol.* **68:**624–641.
55. **Selmecki, A. M., K. Dulmage, L. E. Cowen, J. B. Anderson, and J. Berman.** 2009. Acquisition of aneuploidy provides increased fitness during the evolution of antifungal drug resistance. *PLoS Genet.* **5:**e1000705.
56. **Soll, D. R., and C. Pujol.** 2003. *Candida albicans* clades. *FEMS Immun. Med. Microbiol.* **39:**1–7.
57. **Staib, P., M. Kretschmar, T. Nichterlein, H. Hof, and J. Morschhäuser.** 2002. Host versus in vitro signals and intrastrain allelic differences in the expression of a *Candida albicans* virulence gene. *Mol. Microbiol.* **44:**1351–1366.
58. **Sullivan, D., and D. Coleman.** 1998. *Candida dubliniensis*: characteristics and identification. *J. Clin. Microbiol.* **36:** 329–334.
59. **Sullivan, D. J., G. P. Moran, E. Pinjon, A. Al-Mosaid, C. Stokes, C. Vaughan, and D. C. Coleman.** 2004. Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*. *FEMS Yeast Res.* **4:**369–376.
60. **Sullivan, D. J., T. J. Westerneng, K. A. Haynes, D. E. Bennett, and D. C. Coleman.** 1995. *Candida dubliniensis* sp. nov. phenotypic and molecular characterization of a novel species associated with oral candidosis in HIV-infected individuals. *Microbiology* **141:**1507–1521.
61. **Tavanti, A., A. D. Davidson, M. J. Fordyce, N. A. R. Gow, M. C. J. Maiden, and F. C. Odds.** 2005. Population structure and properties of *Candida albicans*, as determined by multilocus sequence typing. *J. Clin. Microbiol.* **43:** 5601–5613.
62. **Tavanti, A., A. D. Davidson, N. A. R. Gow, M. C. J. Maiden, and F. C. Odds.** 2005. *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. *J. Clin. Microbiol.* **43:**284–292.
63. **Tavanti, A., A. D. Davidson, E. M. Johnson, M. C. Maiden, D. J. Shaw, N. A. Gow, and F. C. Odds.** 2005. Multilocus sequence typing for differentiation of strains of *Candida tropicalis*. *J. Clin. Microbiol.* **43:**5593–5600.
64. **Tietz, H. J., M. Hopp, A. Schmalreck, W. Sterry, and V. Czaika.** 2001. *Candida africana* sp. nov., a new human pathogen or a variant of *Candida albicans*? *Mycoses* **44:**437–445.
65. **van Asbeck, E., K. V. Clemons, M. Martinez, A. Tong, and D. A. Stevens.** 2008. Significant differences in drug susceptibility among species in the *Candida parapsilosis* group. *Diagn. Microbiol. Infect. Dis.* **62:**106–109.
66. **Wrobel, L., J. K. Whittington, C. Pujol, S.-H. Oh, M. O. Ruiz, M. A. Pfaller, D. J. Diekema, D. R. Soll, and L. L. Hoyer.** 2008. Molecular phylogenetic analysis of a geographically and temporally matched set of *Candida albicans* isolates from humans and nonmigratory wildlife in central Illinois. *Eukaryot. Cell* **7:**1475–1486.
67. **Wu, W., S. R. Lockhart, C. Pujol, T. Srikantha, and D. R. Soll.** 2007. Heterozygosity of genes on the sex chromosome regulates *Candida albicans* virulence. *Mol. Microbiol.* **64:**1587–1604.
68. **Wu, W., C. Pujol, S. R. Lockhart, and D. R. Soll.** 2005. Mechanisms of mating type homozygosis in *C. albicans*. *Genetics* **169:**1311–1327.
69. **Zhang, N., A. L. Harrex, B. R. Holland, L. E. Fenton, R. D. Cannon, and J. Schmid.** 2003. Sixty alleles of the *ALS7* open reading frame in *Candida albicans*: *ALS7* is a hypermutable contingency locus. *Genome Res.* **13:**2005–2017.
70. **Zhao, X., S.-H. Oh, and L. L. Hoyer.** 2007. Unequal contribution of *ALS9* alleles to adhesion between *Candida albicans* and human vascular endothelial cells. *Microbiology* **153:**2342–2350.
71. **Zhao, X., S.-H. Oh, R. Jajko, D. J. Diekema, M. A. Pfaller, C. Pujol, D. R. Soll, and L. L. Hoyer.** 2007. Analysis of *ALS5* and *ALS6* allelic variability in a geographically diverse collection of *Candida albicans* isolates. *Fungal Genet. Biol.* **44:**1298–1309.
72. **Zhao, X., C. Pujol, D. R. Soll, and L. L. Hoyer.** 2003. Allelic variation in the contiguous loci encoding *Candida albicans ALS5*, *ALS1* and *ALS9*. *Microbiology* **149:**2947–2960.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch8

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Cell Cycle and Growth Control in *Candida* Species

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An intriguing feature of *C. albicans* growth is the ability to grow in different morphological forms, which is thought to be essential for its pathogenicity. A key question in the field has been, how does a fungal cell alter its cell cycle in response to environmental signals to change its morphology? Signaling pathways clearly regulate responses to environmental cues, which modulate the degree to which different cell shapes appear. In addition, the decision to grow in a highly polarized, somewhat polarized, or isotropic manner differs at each cell cycle stage, a process ultimately regulated by cyclin-dependent kinases (CDKs), the central players in cell cycle progression. In the past decade, we have seen exciting advances in our understanding of how the cell cycle influences *Candida albicans* morphogenesis. At the time of the writing of the last version of Candida *and Candidiasis*, cellular features (e.g., cell-cell attachment, nuclear division site, Spitzenkörper formation, and vacuolar inheritance patterns) that distinguish the different morphologies were being uncovered. Relatively little was known, however, about the molecular mechanisms that give rise to these form-specific features.

Today, we are beginning to understand some of the molecular steps used by cell cycle regulators such as CDKs to regulate morphogenesis. In this chapter, we describe the major *C. albicans* morphologies and our current understanding of the cell biological and cell cycle features that distinguish them. We highlight recent insights into how cell cycle regulators influence the formation of hypha-specific cellular features in particular. One of the most significant results comes from the discovery of a unique, hypha-specific cyclin, Hgc1. Hgc1, transcribed only in the apical-most hyphal compartment and induced by hypha-specific transcription factors, modifies many of the proteins and cellular structures that are responsible for the features that define true hyphal-form growth in *C. albicans*: maintenance of growth polarization at hyphal tips and preservation of cell-cell attachments at sites of septum formation. Furthermore, recent work indicates that alteration of cell cycle dynamics by environmental stress or induction of cell cycle checkpoints dramatically influences cell shape, both by signaling through the same pathways as are involved in the normal hyphal morphogenesis response and by activating cell cycle checkpoints that delay cell cycle progression. Thus, a model is emerging in which morphogenesis signals "build" a particular cellular shape by activating specific cell cycle regulators.

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MORPHOLOGICAL FORMS EXHIBITED BY *CANDIDA*

C. albicans exhibits the greatest number of cellular morphologies relative to other *Candida* species; the five predominant morphological forms are the yeast form, pseudohyphae, true hyphae, chlamydospores, and opaque cells. Non-*C. albicans Candida* species all form yeast cells and pseudohyphae; opaque cells have only been described for *C. albicans*. Interestingly, while both *C. dubliniensis* and *C. albicans* can form true hypae and chlamydospores, they differ in the extent to which they form these two morphologies: *C. albicans* forms hyphae robustly, whereas *C. dubliniensis* more readily forms chlamydospores. This difference is attributed to differential regulation of the transcriptional repressor *NRG1* in each species (93, 122). For example, on Staib agar, expression of *CaNRG1* in *C. dubliniensis* repressed pseudohyphae and chlamydospore formation, while *C. albicans* does not form chlamydopores on Staib agar unless *CaNRG1* is deleted. The properties of yeast and pseudohyphal cells are generally similar across *Candida* species as well as in the Saccharomytina, while hypha-specific mechanisms are known only for *C. albicans* and *C. dubliniensis*. Because morphogenesis and cell cycle regulation have been studied most extensively in *C. albicans*, this chapter is focused primarily on work in *C. albicans*.

Yeast Cells, Pseudohyphae, and True Hyphae

Classic yeast and pseudohyphal morphologies are ellipsoid, with pseudohyphae being more elongated than yeasts (Fig. 1). Both forms propagate by asymmetric budding and are very similar to the yeast and pseudohyphal morphologies that have been well characterized for *Saccharomyces cerevisiae* (74). Pseudohyphae undergo cytokinesis like yeast form cells but tend to remain attached to one another, not

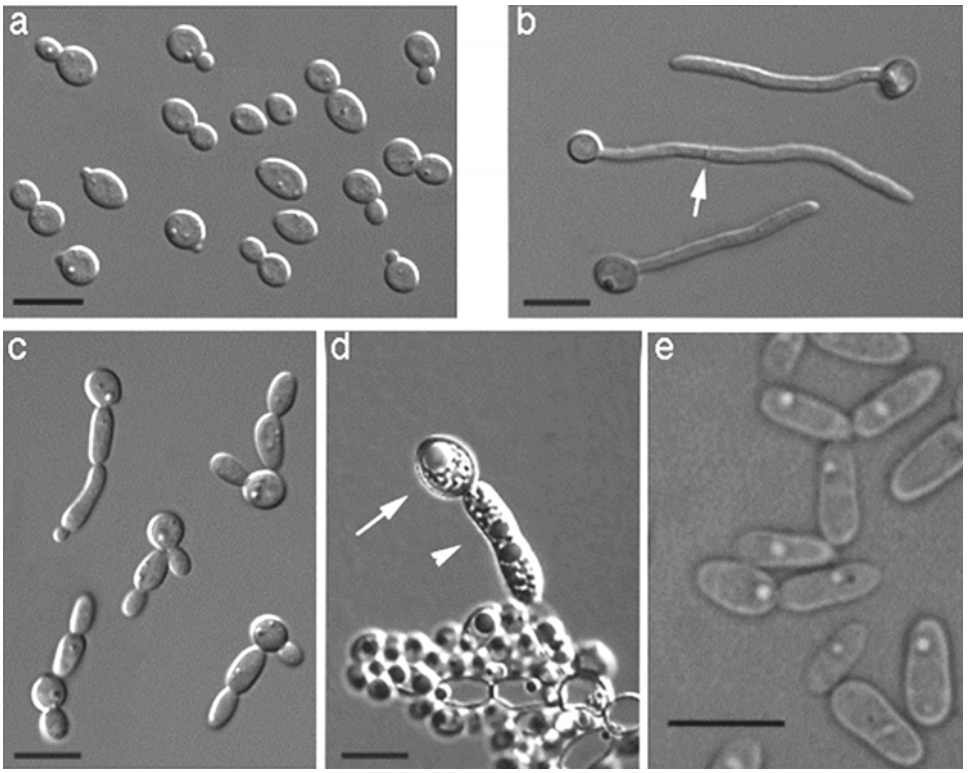

FIGURE 1 Different *C. albicans* morphologies imaged by differential interference contrast microscopy: (a) yeast cells; (b) true hyphae (arrow, septum); (c) pseudohyphae; (d) chlamydospore (arrow) on a suspensor cell (arrowhead); (e) opaque cells. Scale bars, 10 μm. 10.1128/9781555817176.ch8f1

completing the cell separation stage. This results in the formation of chains of cells that appear filamentous, but with obvious constrictions at the bud neck, which are the sites of cytokinesis.

C. albicans yeast and pseudohyphae appear similar to *S. cerevisiae* yeast cells and pseudohyphae, and accordingly, they appear to follow similar cell cycle and cell biological paradigms (Fig. 2). At START, the transition from G_1 to S phase of the cell cycle, yeast buds emerge coordinately with the onset of DNA replication (60). Nuclei divide across the mother-bud neck and cytokinesis is followed by cell separation to form two independent cells, with the mother cell generally being larger than the daughter. As in *S. cerevisiae*, entry into the next cell cycle appears to be dependent on size such that mother cells traverse START before the daughters, a process that makes it difficult to maintain synchrony beyond a single cell cycle.

It is assumed that in pseudohyphae, as in yeast cells, DNA replication initiates at the time of bud emergence. The important distinction between yeast and pseudohyphae is that pseudohyphae spend more time in G_2 phase of the cell cycle than yeast cells (44; reviewed in reference 22), and they continue to elongate during this time. As a result, pseudohyphal daughter buds are not only longer but also larger than yeast form buds. As in yeast cells, nuclei divide across the bud neck. However, as has been observed with *S. cerevisiae* pseudohyphae (74), daughter pseudohyphal cells become similar in size to their mothers, and as a result, the mother-daughter pair often enter the next cell cycle synchronously.

Hyphal growth is induced by many environmental stimuli (97). In particular, a growth temperature of 37°C, the presence of serum, and dilution of stationary-phase cultures into fresh media are efficient inducers of the response. In contrast to pseudohyphae, true hyphae have no constriction at the site of septation. They are slender (≤3 μm in width [107]), parallel sided, and highly filamentous, with elongation rates reported between 11 and 22 μm/h depending on the hypha-inducing media used (13, 54, 58, 107). Hyphal compartments are individual, uninucleate, and demarcated by chitin-containing septa that form perpendicular to parallel sides and without obvious constrictions. Upon hyphal induction and prior to the first septation event, the emerging hyphal evagination is termed a germ tube. Importantly, germ tube evagination, unlike bud emergence, is not coupled to START: cells do not initiate DNA replication or spindle pole duplication at this time; thus, the classic "bud or cell growth" cycle is uncoupled from the "nuclear" and "spindle" cycles in hyphal cells.

There has long been a controversy as to how pseudohyphae are related to true hyphae. Initial models suggested that yeast cells, pseudohyphae, and true hyphae reside along a continuum (97). Later, based on differences in cell cycle dynamics and subcellular structures, it was proposed that pseudohyphae and hyphae represent two distinct morphological states, with pseudohyphae being more like yeast form growth with respect to cell cycle progression and cell biological markers (discussed in more detail below). Recent evidence has pushed the pendulum back to the morphological-continuum model for *C. albicans* (28), based upon the observation that the expression level of the transcription factor Ume6 has a dose-dependent effect on morphogenesis: low levels of Ume6 expression promote growth in the yeast

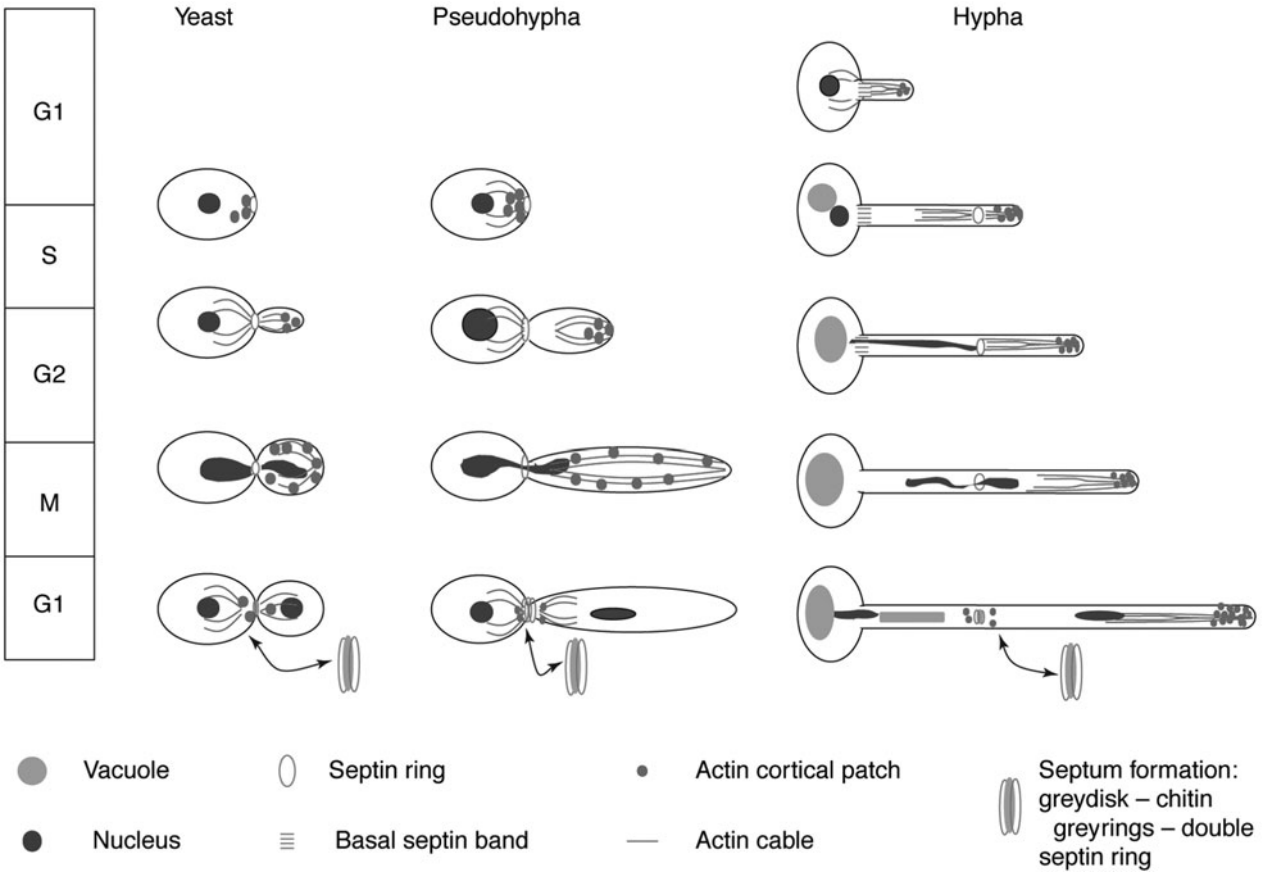

FIGURE 2 Dynamics of organelles and cytoskeletal components during cell cycle progression in yeast and the first cell cycle of pseudohyphae and hyphae following induction of unbudded yeast cells. Hyphal germ tubes emerge prior to the G_1/S transition. The localization patterns of cytoskeletal elements are described in more detail in the text. Reprinted from reference 124 with permission from Elsevier. 10.1128/9781555817176.ch8f2

form, intermediate expression levels give rise to pseudohyphal cells, and high levels of Ume6 expression result in hyphal growth. The idea that pseudohyphae are intermediate between yeast cells and hyphae has led to the proposal that ancient yeast cells used changes in expression levels of key regulatory elements to evolve first to pseudohyphae and then to true hyphae in order to facilitate foraging for nutrients, mating partners, or new growth niches (18).

Importantly, using an in vivo regulatable expression system, increased Ume6 expression levels correlated not only with increased hyphal growth but also with enhanced tissue invasion and virulence in a mouse model of invasive candidiasis (28). The same regulatable system was also used to study the role of the hyphal repressor Nrg1 and gave similar results: *C. albicans* strains derepressed for Nrg1 expression, and in effect unable to form hyphae, had decreased virulence (105). These results strengthen the long-held theory that hyphal growth (or other functions positively regulated together with hyphal growth) is important for the pathogenesis of *C. albicans* infections and, in particular, for host tissue damage and injury (reviewed in references 43 and 148).

Chlamydospores and Opaque Cells

Chlamydospores are thick-walled, nucleated spherical cells that are much larger than yeast cells and that form in media containing complex carbohydrates (2, 27, 89, 92, 135) (Fig. 1). To date, their biological role remains unclear. Early studies note that they germinate by budding (67, 103, 111, 131) and lose this ability as they age, although no recent work has documented chlamydospore germination. Their cell walls are composed of a thin, glucan-rich outer layer and thick protein-rich inner layer (67, 108). Young chlamydospores contain organelles, including a large vacuole, similar to yeast cells (108), and these are lost as the chlamydospore ages. Chlamydospores arise either on hyphal filaments or, more commonly, from the tip of an elongated cell known as a suspensor cell, which branches from a hypha. Despite their association with filamentous cells, hyphal formation is not required for chlamydospore development (11, 121, 126). Recent work has shed light on cell biological features associated with cell cycle progression in chlamydospores and is discussed below.

Opaque cells were discovered by Slutsky and colleagues, approximately 13 years ago, as an alternative phenotype in strain WO-1 (113). More recent work reached the important insight that these cells are highly competent for mating and arise at high frequency only in *C. albicans* strains that are homozygous for the mating type-like loci (88). Opaque cells form flat colonies with a matte, translucent color, as opposed to the dome-shaped white colonies. They are more

oblong than yeast cells and exhibit "pimples" on their surface (reviewed in references 116 and 119). The switch from the white (classic yeast) to opaque phase not only facilitates mating but also influences the pathogenesis of *C. albicans* infections (reviewed in references 19, 96, and 117). No studies to date have analyzed the spatial and cell cycle-mediated regulation of morphogenesis in opaque cells. The frequency of white-opaque phase switching increases when cell cycle progression is slowed in general. For example, exposure of cells to genotoxic stresses or depletion of the Clb4 mitotic cyclin results in a 20- to 50-fold increase in the rate of opaque cell generation, without affecting the level of protein synthesis (3). This result is thought to be due to the opaque-promoting transcription factor Wor1, which is able to accumulate to higher levels when cells grow more slowly.

CELL BIOLOGICAL FEATURES THAT DISTINGUISH *CANDIDA* MORPHOLOGIES

Because cell wall deposition is largely permanent, fungal cell shape is intimately linked to cell cycle progression and the degree to which cell cycle regulation prescribes the relative proportion of time a cell spends in polarized versus isotropic growth. While the ideal situation would allow study of morphogenesis using conditions that promote 100% yeast, pseudohyphal, or hyphal cells within the population, in practice, a single culture or histological specimen of *C. albicans* often includes yeast, pseudohyphal, and hyphal forms concurrently. In addition, some cells exhibit morphologies that appear to be intermediate between the classically defined morphological forms. For example, elongated pseudohyphae can appear almost hyphal. An objective characterization of cell shape that has stood the test of time is the "morphology index" (Mi) (87), in which maximal cell length, cell width, and width at the site of septation are used to describe individual cells (Fig. 3). Mi values range from 1, for completely spherical cells, to ~4, for true hyphal filaments (Table 1). Exciting, recently developed alternative strategies to characterize morphology include the use of soft X-ray tomography to distinguish *Candida* growth forms based on differences in subcellular characteristics (127). A different approach has extended more quantitative analysis of conventional phenotypes using an impressive high-throughput analysis of colony growth and morphology, the latter of which is dependent upon the proportion of cells forming different morphologies within the population. This was recently applied to characterize phenotypes for a large collection of mutants that each had deletions of a single transcription factor and analyzed over a range of 55 growth conditions (62). High-throughput image collection and analysis of individual cell morphologies, using the Mi or some analogous method, would extend the study of morphogenesis regulation to the individual cell level.

Of course, it is the underlying cell biology, including cytoskeletal features and other structural components of the cell, that determines the direction of deposition of cell wall material and, ultimately, cell shape. Thus, it is critical to understand the cell biological mechanisms that define and distinguish the different cell morphologies. The cell biological characteristics that differentiate *C. albicans* morphologies, including bud site selection patterns, microtubule and mitotic spindle organization, actin cytoskeleton dynamics, formation of structures required for polarized growth (the polarisome and/or Spitzenkörper), vacuolar inheritance mechanisms, and patterns of septation and cell separation, are detailed below and are summarized in Table 1.

Bud Site Selection Patterns

The pattern of bud site selection is determined by staining for chitin that is enriched within the cell wall formed around sites of septation. In *S. cerevisiae* yeast cells, bud site selection follows a highly ordered pattern based on the nuclear ploidy of the yeast cell: haploid cells bud with an axial pattern, with the bud emerging at sites adjacent to the previous bud site; diploid cells bud with a bipolar pattern, emerging either adjacent to or opposite from the previous bud site. In contrast, pseudohyphal cells bud with a unipolar pattern, with buds emerging at the distal pole such that progeny reach farther and farther distances from the original cell.

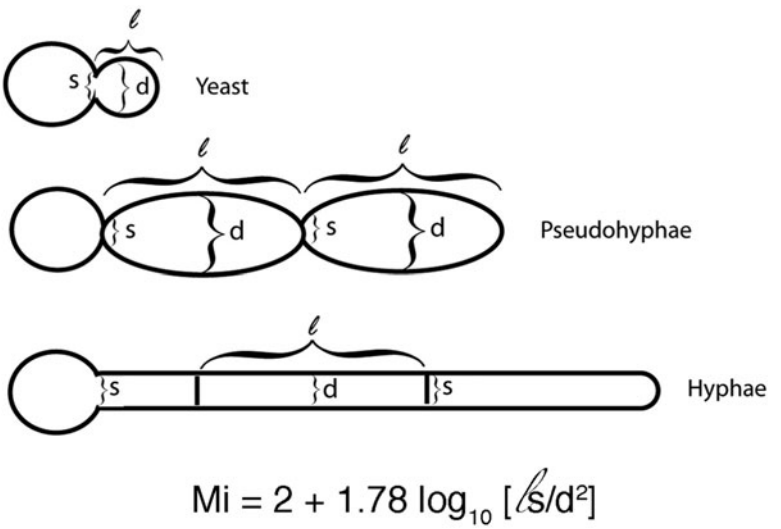

FIGURE 3 Method of calculation of the Mi (87). *l*, length; d, maximum diameter; s, diameter at mother-daughter cell junction. The morphology ratio (*l*/d) has a value near 1.0 in a sphere and becomes larger in a more filamentous cell. 10.1128/9781555817176.ch8f3

TABLE 1 Cell biological features of *Candida albicans* morphologies[a]

Feature(s)	Yeast	Pseudohypha	Hypha	Chlamydospore
Shape and septation	Ellipsoid Constriction at septum	Elongated ellipsoid Constriction at septum	Parallel-sided filament No constriction at septum	Large, spherical No septum
Propagation	Budding	Budding	Elongation from tip of primary hypha; hyphal branching	Unclear
Mi	1.0–2.5	2.5–3.4	>3.4	1.0–1.5
Bud site selection	Axial and bipolar patterns which are temperature dependent	Unipolar pattern	Random sites of germ tube emergence that can be overridden by external cues	Not described
Vacuolar inheritance	Relatively symmetric	Relatively symmetric	Asymmetric	Not described
First septum	At mother-daughter neck	At mother-daughter neck	Within germ tube	At suspensor-spore neck
Actin patches	Cycles of polarized and isotropic localization dependent on cell cycle	Cycles of polarized and isotropic localization dependent on cell cycle	Constitutively polarized to hyphal tip independent of cell cycle	Not described
Microtubules	Required for nuclear segregation at mitosis	Required for nuclear segregation at mitosis	Required for apical growth and nuclear segregation at mitosis	Not described
Septin structure	Patch at incipient bud site; ring at mother-daughter neck prior to cytokinesis	Patch at incipient bud site; ring at mother-daughter neck prior to cytokinesis	Band-like structure at mother-germ tube neck; ring at presumptive septum	Young cells: ring at suspensor-spore neck Older cells: long, elaborate filaments at plasma membrane
First nuclear division site	Across septum at mother-daughter junction	Across septum at mother-daughter junction	Across septum within germ tube; retrograde movement of one nucleus back to mother cell	Within suspensor cell; daughter nucleus migrates forward into chlamydospore
Polarisome	Crescent-shaped cap at bud apex; includes Bud6, Spa2, Bni1, Mlc1	Crescent-shaped at bud apex; includes Bud6, Spa2, Bni1, Mlc1	Crescent at hyphal apex; includes Bud6, Spa2, Cdc42, Cdc24	Not described
Spitzenkörper	None	None	Spherical, just behind tip apex; includes vesicles, Bni1, Mlc1; required for polarized cell shape and for growth directionality	Not described

[a]Detailed descriptions and references are found in the text.

In *C. albicans*, which is an obligate diploid, yeast bud site selection is temperature dependent. Cultures generally contain a mixture of cells that exhibit axial and bipolar bud scar patterns, with more axially budding cells at lower temperatures (e.g., <30°C) and a more equal mixture of cells with axial and bipolar patterns appearing at higher temperatures (e.g., ≥30°C) (29, 47). The budding pattern of *C. albicans* pseudohyphae is like that seen in *S. cerevisiae*: unipolar patterns result in branching filaments that extend distally away from the original cell.

Maintenance of budding patterns is executed by a set of landmark proteins that direct polarized growth factors to discrete sites on the mother cell cortex. In *S. cerevisiae*, Bud3 and Bud4 are necessary for axial budding patterns, and when they are lost, cells revert to a bipolar budding pattern (30). The bipolar budding pattern requires a number of other bud site selection proteins, including Bud6, a protein that nucleates actin cables and interacts with both actin and formin proteins to mediate polarized growth (4), and the Bud1/Rsr1 GTPase together with its GTPase-activating protein (GAP), Bud2 (30, 99). At least some of the landmark mechanisms present in *S. cerevisiae* appear to operate in *C. albicans*. In *C. albicans*, Bud4/Int1 also functions as an axial marker. Deletion of the gene encoding Bud4/Int1 increases the proportion of cells with the bipolar budding pattern (47), suggesting that, as in *S. cerevisiae*, the axial pattern is a modification of the bipolar pattern. Despite this, there are no obvious homologs of the *S. cerevisiae* bipolar positioning landmarks (Bud8 and Bud9), suggesting that an alternative bipolar bud site selection mechanism exists and remains, as in *S. cerevisiae*, epistatic to the axial budding process. Similar to the case with *S. cerevisiae*, loss of *C. albicans* Bud6 (120) or of the Bud1/Rsr1 GTPase module, including Bud1/Rsr1 and/or the Bud2 GAP for the GTPase, results in random budding patterns (58, 142), indicating that these cells have lost the ability to place a landmark in the cell. Ultimately, the landmark must recruit Cdc42, the GTPase that coordinates polarized growth. An elegant study of bud site selection under such conditions includes a mathematical model that explains why cells lacking any landmark are still programmed to form only one bud: a positive-feedback loop concentrates clusters of Cdc42 and the proteins it recruits to a region that nucleates polarized growth; simple and rapid competition for additional Cdc42 leads to only one of these sites "winning" the competition (64).

During hyphal growth in liquid cultures, germ tubes emerge from either polar or random locations on the mother cell with respect to previous bud scars (61), suggesting that, while tumbling in an environment without directional signals, cells may not utilize a landmark system for selecting sites for germ tube emergence. In contrast, germ tubes do emerge from the mother cell in a directional manner when they are exposed to external stimuli such as cationic charge on a solid substrate (polylysine-coated slide) (37). Importantly, this response to the direction of a signal requires calcium channels (23) and the landmark GTPase Rsr1 (24). Similarly, reorientation responses in neuronal growth cones require the asymmetric localization and activation of Rho-type GTPases and in some cases are mediated by intracellular calcium gradients (56, 101, 102). Thus, analogous molecular mechanisms appear to mediate directional growth responses in elongated cells of fungi and vertebrates.

Microtubules and Spindle Dynamics

During cell cycle progression, microtubules that emanate from spindle pole bodies mediate nuclear orientation perpendicular to the axis of the bud neck and nuclear migration to the site of septation. In *C. albicans* yeast cells and pseudohyphae, the spatial and temporal aspects of microtubule dynamics, including spindle pole body duplication and mitotic spindle elongation, resemble those established for *S. cerevisiae*. In *C. albicans* hyphae, there are two challenges to nuclear migration. First, nuclei travel longer distances to reach the site of septation than in yeast or pseudohyphal cells, and second, after nuclear division, the mother nucleus moves in a retrograde fashion in order to return to the mother cell. Several solutions contribute to overcome these challenges. First, in hyphae, mitotic spindles are much longer (12 to 20 μm) than in yeast cells (7 to 8 μm) (16), and second, prior to mitosis, nuclei migrate far into the germ tube, powered primarily by the motor protein dynein, which is localized at the cell cortex and pulls on the lateral surface of astral microtubules (45). The retrograde movement of the mother nucleus after mitosis occurs primarily in a dynein-independent manner that first occurs relatively rapidly as a consequence of spindle elongation and later continues more slowly through the activity of other motor proteins (44). In addition to their role in nuclear cell cycle dynamics, microtubules also control morphogenesis through their regulation of the Spitzenkörper (literally, "tip body"; described below).

Actin Patches and Cables

The actin cytoskeleton is required for cell growth in all *C. albicans* cell types (1, 6). In fungi such as *S. cerevisiae* and *C. albicans* the actin cytoskeleton is composed of two structures: actin patches that are primarily located at the cell cortex and cytoplasmic cables that usually direct vesicular traffic toward sites of growth. Actin patches are concentrated at sites of polarized growth, where they function in endocytosis to mediate vesicle invagination and movement to the interior of the cell (70, 94). In all cell types, actin cables serve as railways for the directed secretion of cargo to the daughter cell apex and are required for polarized growth of yeast and pseudohyphal buds and for the elongation of hyphal tip cells (1, 36, 143). Actin cables function together with the actin patches to recycle cortically localized polarity proteins, preventing them from diffusing too far from the cell front and ensuring the maintenance of their polarized distribution at daughter cell tips (66).

The cell cycle dynamics of actin patch and cable localization are illustrated in Fig. 2. Just before the emergence of either a bud (at G_1/S) or a germ tube (prior to G_1/S), actin patches cluster at the site of evagination and then remain polarized at the apex of the growing daughter cell. In yeast cells, actin patches are redistributed isotropically throughout the daughter cell cortex at G_2/M. This shift to isotropic growth (and from G_2 to M) occurs later in pseudohyphae than in yeast cells (22, 36, 74; K. Finley and J. Berman, unpublished data), which contributes to the potential for longer and larger daughter cells. Finally, after mitosis, actin patches carrying cargo necessary for cytokinesis and cell separation relocalize to the incipient septum. Actin dynamics are distinctively different in hyphal cells: the majority of actin patches remain polarized constitutively to the hyphal tip irrespective of the cell cycle stage. In this sense, again, hyphal cell growth is uncoupled from nuclear and spindle events.

In addition to the structural role of actin in the cytoskeleton, monomeric (G) actin is proposed to be a part of a sensor/effector apparatus that mediates hyphal induction signals (150). Intriguingly, G-actin copurifies with adenyl cyclase (Cyr1), the enzyme responsible for cyclic AMP

(cAMP) synthesis, and its associated protein Cap1, which is necessary for full activation of Cyr1. A purified complex containing Cyr1, Cap1, and G-actin is sufficient for production of a spike in cAMP synthesis in response to hyphal induction signals. In contrast, complexes lacking G-actin or Cap1 have significantly attenuated Cyr1 activity. Furthermore, actin toxins (latrunculin A and cytochalasin A) inhibit cAMP synthesis by the purified Cyr1–Cap1–G-actin complex without causing dissociation of actin from the complex. Together, these results are consistent with the idea that the conformation of G-actin and its interaction with Cyr1 and Cap1 are required for the production of cAMP in response to hyphal induction signals (150). Importantly, these results provide a mechanism whereby *C. albicans* can transduce a cytoskeletally sensed signal directly to the cAMP/protein kinase A signaling pathway to quickly activate hyphal morphogenesis.

Polarizing Structures: the Polarisome and Spitzenkörper

In yeast and pseudohyphal buds, polarized secretion is targeted to a crescent-shaped region of the bud apex called the polarisome. Proteins known to comprise the polarisome and to direct actin-mediated secretion include Bud6 and Spa2 (4, 36, 109, 115). In addition, actin nucleation factors such as the formin-containing protein Bni1 and the myosin light chain protein Mlc1 colocalize with polarisome components at bud tips (36, 98, 100). The polarisome complex and its interaction with actin cables are regulated by the Rho-type GTPase Cdc42, which localizes to the bud cortex and is essential for polarized growth (59, 149). Like the actin cytoskeleton, polarisome proteins in yeast and pseudohyphal cells reorganize during cell cycle progression: once bud growth is complete (following M phase), they relocalize to the septum, where they direct secretory vesicles to the site of cytokinesis (4, 83, 98, 115).

A striking feature of hyphal cells is their ability to form extremely narrow, highly polarized daughter cells. The size of the region to which secretion is targeted on the daughter cell cortex is a critical property that dictates cell shape. In *C. albicans* hyphae, two tip-localized entities influence hyphal elongation and shape: the polarisome and a fascinating structure known as the Spitzenkörper. The Spitzenkörper, first described for the filamentous fungus *Polystictus versicolor* (50), is a three-dimensional vesicle-rich structure just behind the hyphal tip of actively growing cells that is readily detected using the lipophilic dye FM4-64 (Fig. 4a) (36). Currently, it is not clear if the Spitzenkörper is composed of a specific type of endosome or of subpopulations of functionally distinct endosomes.

The Spitzenkörper directs polarized hyphal growth by virtue of its proximity to the hyphal tip, which enables secretory vesicles to reach a small surface area of the hyphal tip with high frequency. Mathematical modeling of polarized growth for several filamentous fungi demonstrated that the concentration gradient of vesicles provided by the Spitzenkörper closely predicts the hyphal tip shape (15). By dictating the secretory path of a hypha, the Spitzenkörper likely guides the direction of *C. albicans* hyphal elongation. For example, changes in growth directionality can be anticipated by repositioning of the Spitzenkörper in many filamentous fungi (82, 104).

The molecular characteristics of the Spitzenkörper, its relationship with the polarisome, and how they both contribute to hyphal morphogenesis are now beginning to become clear. For example, Bud6 localizes predominantly to a polarisome-like crescent at the tip of hyphae (Fig. 4c and d) (36) as in yeast cells. In contrast, Bni1 and the myosin light chain protein Mlc1 colocalize with FM4-64-stained Spitzenkörper vesicles (Fig. 4b) (36) in hyphae and, in contrast, with the polarisome in yeast cells. In addition, treatment of hyphae with drugs that disrupt microtubules disrupt the Spitzenkörper localization of Mlc1, but not its ability to localize to the polarisome (36). Spa2, a polarisome component in yeast cells, localizes to a small extent to the Spitzenkörper and to a greater degree in the polarisome-

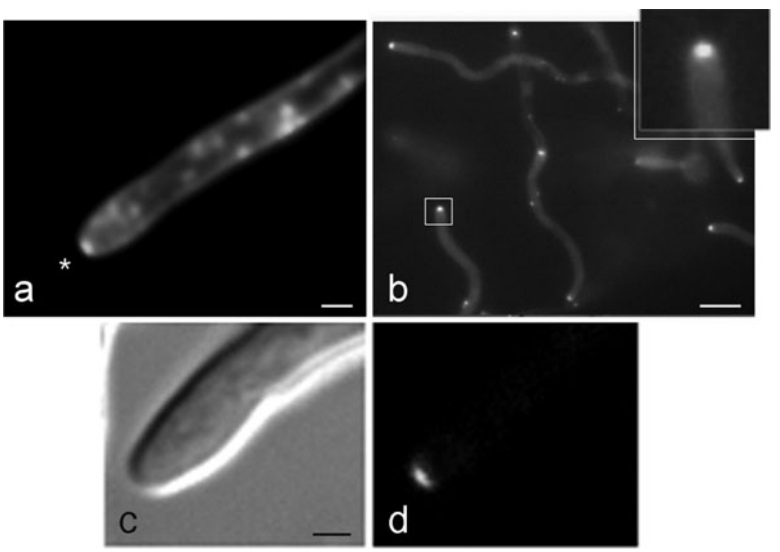

FIGURE 4 Spitzenkörper (a and b) and polarisome (d) structures at *C. albicans* hyphal tips. FM4-64-stained vesicles (a, asterisk) and Mlc1-YFP (b) localize apically to a spherical body. Polarisome-associated protein Bud6 tagged with YFP localizes in a crescent at hyphal tips (d; corresponding differential interference contrast image is in panel c). Scale bars, 2 μm (a), 10 μm (b), and 1 μm (c). Inset (b, square), magnification of ×5. 10.1128/9781555817176.ch8f4

like crescent of hyphae (36). In addition, Cdc42 and its guanine nucleotide exchange factor, Cdc24, which are essential for all modes of polarized growth and are present in higher concentration at hyphal tips, localize in a polarisome-like fashion in hyphae (17, 59) as well as at the bud tips of yeast cells and pseudohyphae. Together, these results imply that different mechanisms recruit proteins to the two different structures in yeast cells and hyphae. While the polarisome and Spitzenkörper appear distinct and are enriched in different proteins, they are not completely independent structures. Deletion of either of the polarisome-associated proteins Bud6 or Spa2 causes the Spitzenkörper-associated Mlc1 protein to relocalize to a polarisome-like crescent pattern and results in less polarized hyphal tip growth (36).

A very important feature of hyphal cells is that cell elongation is constitutive and proteins associated with both the polarisome and the Spitzenkörper localize persistently to the tip (17, 36, 59, 77, 84, 147). This cell cycle-independent maintenance of at least one polarity protein, Spa2, requires the Bud1/Rsr1 Ras-like GTPase (58). In contrast, in yeast and pseudohyphal cells, polarisome proteins relocalize from the bud apex to the cytokinetic ring in a cell cycle phase-dependent manner (17, 36, 59, 77, 84, 147). However, some of these proteins also appear at the site of septation following mitosis in hyphae. It remains to be determined if they are a subset of proteins that relocalized from the elongating tip or if they are newly produced proteins that are recruited directly to the site of septum formation.

Vacuolar Inheritance Mechanisms

In contrast to the relatively symmetric inheritance of vacuoles during yeast and pseudohyphal growth, *C. albicans* hyphae exhibit asymmetric vacuolar inheritance that correlates with modulations of the cell cycle during growth (13). Parental cells (mother yeast or subapical hyphal compartments) inherit large vacuoles and a relatively small proportion of cytoplasm after cytokinesis; conversely, elongating daughter compartments (apical hyphal cells, secondary germ tubes, or hyphal branches) are largely devoid of vacuoles and inherit a majority of the cytoplasm (Fig. 5) (53, 55). It is thought that the relatively small proportion of cytoplasm in subapical compartments limits the ability of cells to traverse START and reenter the cell cycle. Thus, they remain paused in G_1, sometimes for several cell cycles (13, 52, 59, 74). This quiescent state of subapical cells underlies the linearity of hyphal growth rates, as more relative branch or bud formation would result in faster growth. Once subapical cells have accumulated a critical increase in cytoplasmic volume and, in parallel, a decrease in vacuolar volume, they are able to traverse START and reenter the cell cycle by initiating the emergence of a new hyphal branch (Fig. 5) (13). Importantly, mutations in vacuolar formation and segregation affect the frequency of hyphal branching (14, 133, 134), supporting the idea that vacuolar inheritance patterns are intimately linked to cell cycle progression.

Septins, Septation, Nuclear Division, and Cytokinesis

In *C. albicans*, septin structures (10-nm filament rings) were first observed in yeast cells and hyphae by transmission electron microscopy (118). In yeast cells and pseudohyphae, septin proteins (Cdc3, Cdc10, Cdc11, Cdc12, and Sep7) initially form a patch at the incipient bud site that then grows into a single ring-like structure at the mother-bud neck and that eventually splits into two parallel ring-like structures after mitosis (Fig. 2 and 6A) (125, 138). The process appears to be analogous to septin formation and separation in *S. cerevisiae* (80). The septin ring acts as a scaffold for proteins that ensure proper spindle orientation, nuclear transit through the neck, the timing and position of nuclear division, and the recruitment of polarized growth factors required for septation, cytokinesis, and cell separation.

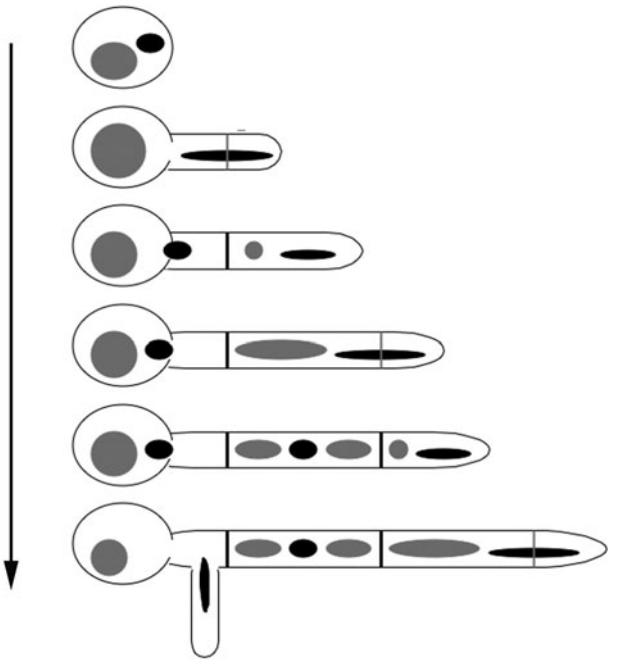

FIGURE 5 Relationship between vacuolar inheritance and hyphal elongation during cell cycle progression. Cell cycle progression is noted by the arrow to the left. Prior to mitosis, the parental vacuole enlarges relative to the total cell volume. Subapical hyphal cells contain large vacuoles and pause at G_1 phase; apical cells have smaller vacuolar volumes (and thus a higher volume of cytoplasm) and actively progress through the cell cycle. Prior to branching (reentry into the cell cycle [bottom panel]), subapical cell vacuoles become smaller and the volume of cytoplasm becomes larger relative to the total cell volume. Gray, vacuole; black, nucleus; black line, septum; gray line, septin ring ("presumptum"). Adapted from references 124 and 13 with permission from Elsevier and the American Society for Microbiology. 10.1128/9781555817176.ch8f5

A critical difference between yeast cells and pseudohyphae is that pseudohyphae do not undergo cell separation following cytokinesis, at least in part because they produce less chitinase, the enzyme that degrades cell wall material that holds daughter cells together. Indeed, strains with decreased expression of chitinase and endoglucanase enzymes exhibit pseudohyphal morphologies when grown in yeast form-promoting media (38, 39, 71, 106, 137).

In hyphal cells (Fig. 2 and 6B), septins do not form a ring at the mother-filament neck. Rather, they form a "basal septin band" structure (125), which differs from a septin ring in that it neither includes septin Cdc3 (58) nor prescribes the site of nuclear division. Rather, a true septin ring, including Cdc3, forms within the elongating germ tube and marks the site of eventual nuclear division, the so-called "presumptum" (44, 125) that goes on to form the chitin-containing septum. As in yeast cells and pseudohyphae, septin rings in hyphae recruit factors important for cytokinesis and septum

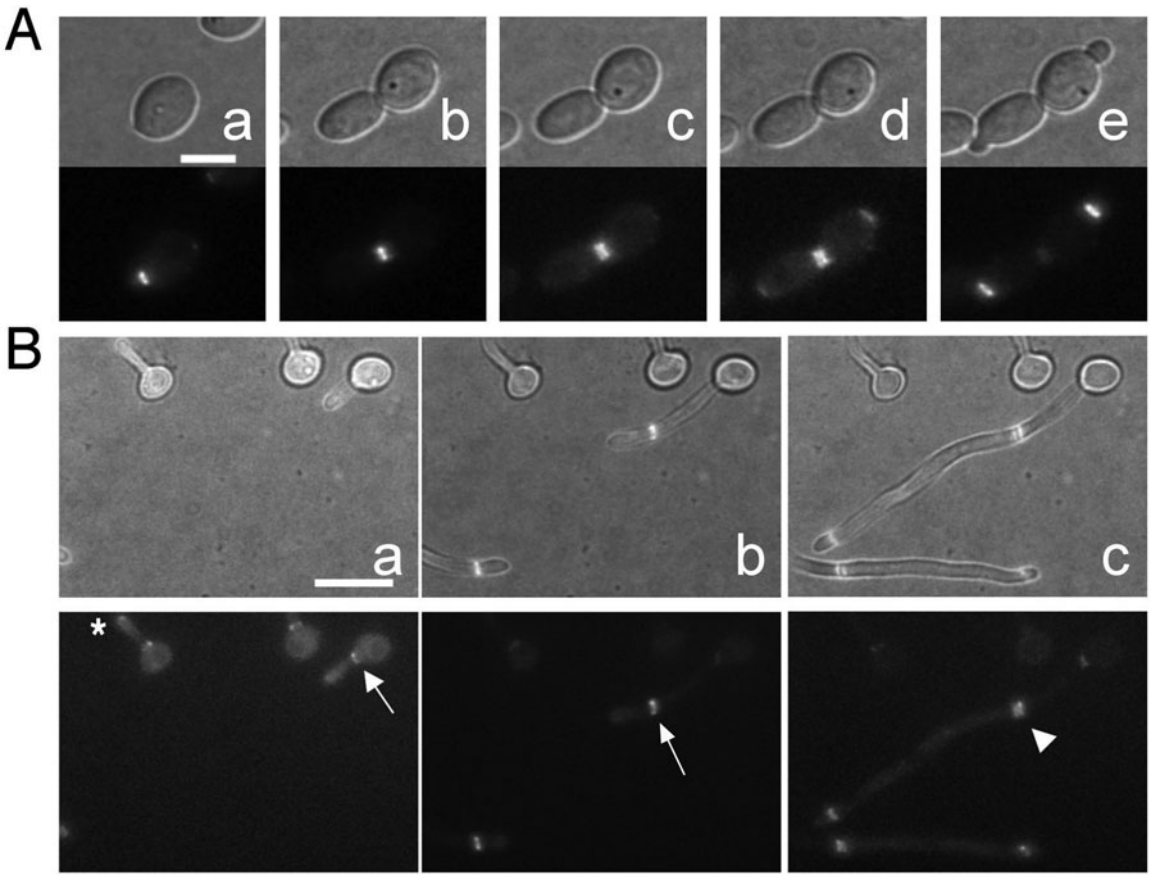

FIGURE 6 Septin localization during the C. *albicans* cell cycle. Shown are time-lapse differential interference contrast (top panel of each set) and fluorescence (bottom panel of each set) images of C. *albicans* cells expressing a Cdc10 septin-yellow fluorescent protein fusion protein. In pseudohyphae (A), a septin cap localizes to the presumptive pseudohyphal bud site in G_1 phase (a). Throughout the S and G_2 phases, the septins organize into a collar at the mother-bud neck (b). At mitosis, the septin collar splits into two rings (c). Cytokinesis occurs between the two rings, the rings disassemble, and the new septin cap appears during the next G_1 (d and e). Bar, 5 μm. In hyphae (B), as the germ tube emerges, a septin spot localizes to the hyphal tip (a, asterisk) and a basal septin band is visible at the mother-daughter neck (a, arrow). The classic septin ring marks the future site of septation (the presumptum; b, arrow). At later time points, the septin ring splits into two rings (c, arrowhead). Bar, 10 μm. 10.1128/9781555817176.ch8f6

formation. However, hyphal septa exclude factors, such as the Cdc14 phosphatase, that are necessary for cell separation (51; discussed in more detail below).

In chlamydospores, a septum, prescribed by a septin structure, forms between the chlamydospore and suspensor cell, but the cells remain attached. Interestingly, nuclear division occurs in a unique manner: nuclei first divide within the suspensor cell and not across a septin structure. Subsequently, the daughter nucleus migrates across the neck into the young chlamydospore (85). In older chlamydospores, the septins form elaborate filamentous structures at the plasma membrane that may play a role in cell membrane stability and strength (85).

CYCLIN REGULATION AND MORPHOGENESIS

Temporal and spatial oscillations in the level and activity of specific cyclins and their interaction with a CDK (Cdc28 in both S. *cerevisiae* and C. *albicans*) regulate cell cycle progression events, including the timing of DNA replication (S phase) and division (M phase) as well the shifts between polarized and isotropic growth. In addition, at least under some conditions, signals and regulators can override the basic cell cycle instructions that lead to the characteristic shapes of the different morphological forms. It appears that the C. *albicans* morphogenesis machinery is exquisitely sensitive to many different signals and regulators as well as to conditions that delay cell cycle progression: a vast majority of stress conditions causes some change in cell shape, which is often evident in different colony morphologies as well (reviewed in references 22 and 139).

A limitation to the study of cell cycle progression has been the relative difficulty in obtaining good cell cycle synchrony. Cultures can be manipulated to generate a relatively synchronous exit from stationary phase to hyphal induction (60, 125), and elutriation of small, unbudded cells (reviewed in reference 46) has been used to synchronize yeast growth

in *C. albicans* (20, 79; M. McClellan, B. Futcher, and J. Berman, unpublished results). Use of mutants and drugs to generate synchrony is complicated by the fact that cell cycle arrest is usually accompanied by continued growth to form cells with a pseudohyphal-like morphology. Recent construction of a *C. albicans MTL***a** strain that overexpresses Far1, a CDK inhibitor that causes rapid and efficient late G_1 arrest of the cell division cycle (34), was exploited to generate synchronous cultures for analyzing gene expression profiles (33). Oscillation of cell cycle genes was detected despite the fact that fewer than 50% of the cells retained good synchrony by completion of the first cell cycle. Nonetheless, the majority (~80%) of periodically expressed genes in *C. albicans* have homologs in *S. cerevisiae* (33), which is not surprising given the central role of cell cycle regulation in all eukaryotes.

In the *C. albicans* genome sequence, there are three G_1 cyclins (Ccn1, Cln3, and Hgc1) and two G_2 or B-type/mitotic cyclins (Clb2 and Clb4) that are predicted to associate with Cdc28 (Table 2). The presence of Clb1 and Clb3 in *S. cerevisiae* and not in *C. albicans* can be attributed to the whole-genome duplication (WGD) event that occurred in the *S. cerevisiae* lineage (141). Intriguingly, *C. albicans* has no obvious homolog of either Clb5 or Clb6, the pair of *S. cerevisiae* cyclins that are required to regulate DNA replication. This implies that DNA replication timing may be regulated differently in these two yeasts. Indeed, replication dynamics do have some significant differences in *C. albicans*: centromeres all have an associated origin, a feature conserved with *Yarrowia lipolytica* (132) and several other yeasts, and centromere DNA is replicated earlier than any other region of the genome, a feature that appears to be shared with *Schizosaccharomyces pombe* as well (72, 73). Below we discuss the role of the classic G_1 and mitotic cyclins. We then highlight exciting new work concerning cyclins that are not strictly cell cycle regulators: the hypha-specific Hgc1 as well as Pcl1 proteins that interact with the Pho85 kinase.

Importantly, upon hyphal induction, germ tube emergence from a G_0 yeast cell occurs prior to the expression of G_1 cyclins (Fig. 2) (60, 124). Nonetheless, it has been unclear whether hyphal growth can be induced in cells at all cell cycle stages. In early condition shift studies, Mitchell and Soll concluded that hyphal growth can only be initiated from yeast cells prior to the point at which they switch from polarized growth to isotropic growth, which is some time in S/G_2 (90). In contrast, in 2002, Hazan and colleagues analyzed cells shifted to hyphal induction and observed "bottlenose"-shaped cells that emerged from a wider base, suggesting that hyphal growth can be initiated at any time during the yeast cell cycle, even in cells that have made the switch to isotropic growth (60). In the latter study, however, it was unclear if growth was truly hyphal or just polarized in nature. In support of the latter model, Zheng and Wang reported that expression of the hypha-specific G_1 cyclin Hgc1 can be induced in cells arrested at S and at M that were grown in hypha-promoting media (serum), suggesting that Hgc1 (and the hyphal program) can be activated in yeast cells at different stages of the cell cycle (145). Some of the uncertainty surrounding this issue involves the distinctions between, and definitions of, polarized growth and hyphal cells. These could be resolved by analyzing hypha-specific cellular features (presence of a Spitzenkörper, localization of septins at mother-filament necks, and site of nuclear division) in these polarized cells. What is clear is that hyphal induction conditions, especially strong ones like serum at 37°C, can induce cells to grow in a much more polarized manner.

G_1 Cyclins

G_1 cyclins are responsible for the transition from G_1 phase to S phase, and in *S. cerevisiae*, Cln3 regulates the transcription of the other two G_1 cyclins, Cln1 and Cln2, such that their mRNA levels peak in late G_1 (123). Similarly, in *C. albicans*, the G_1 cyclins Ccn1 and Cln3 peak during G_1/S (initiation and polarized growth of buds) and decrease by G_2/M (isotropic growth of buds and septation), corresponding to the time at which the B-type cyclins peak (Fig. 7) (21, 22, 60, 79). However, the role of Cln3 is quite different in *C. albicans*. First, *CaCLN3* is essential, albeit for yeast growth and not for hyphal-form growth. This distinction underscores the idea that cell cycle regulation mechanisms differ dramatically between yeast and hyphal cells and that hyphal cell cycle progression is regulated through mechanisms that do not operate in *S. cerevisiae*. For example, *C. albicans* yeast cells lacking Cln3 increase in size to form giant cells that do not bud; subsequently they initiate hyphal growth (9, 31). The mechanism for this intriguing phenotype remains unclear, but it implies that Cln3 regulates polarized growth in yeast cells but not in hyphal cells and that Cln3 function must be regulated, in turn, by one or more cell-type-specific factors. However, Cln3 mRNA levels did not exhibit periodic expression in this cell cycle transcription profiling experiment (33). What remains unclear is whether Cln3 is cell type specific and expressed only in yeast cells and not in hyphal cells. However, some array experiments do detect Cln3 mRNA during growth at 37°C (41), suggesting that Cln3 regulation of cell type does not occur exclusively at the level of transcription.

Ccn1, a second G_1 cyclin, is not essential for yeast growth but is important for yeast cell cycle progression. Unbudded yeast cells lacking Ccn1 enter the cell cycle early yet take longer than wild-type cells to complete a single cell cycle (79). The large size of *ccn1* null cells is likely due to the longer time they spend in a single cell cycle; it most likely contributes to the early entry into the next cell cycle, since cell size is a major factor affecting the relative timing of the G_1/S transition (68, 79). Of note, in strains lacking Grr1, a G_1 cyclin degradation factor, cells form pseudohyphae in yeast-promoting media and are still able to form hyphae in response to hyphal induction conditions (78). Thus, consistent with the idea that G_1 cyclins promote polarized growth in yeast cells but are not essential for hyphal morphogenesis, the persistence of G_1 cyclins is associated with hyperelongation under yeast growth conditions, yet reducing the levels of G_1 cyclins does not inhibit hyphal morphogenesis. Strikingly, of the 20% of genes that exhibit a peak of expression early in the cell cycle and do not have an obvious *S. cerevisiae* homolog, the majority of them exhibit peak expression during G_1/S or M/G_1. This implicates G_1 as a critical stage of the cell cycle for processes such as hyphal morphogenesis that occur in *C. albicans* and not in *S. cerevisiae*.

During the first cell cycle following hyphal growth induction, both G_1 cyclins (Cln3 and Ccn1) persist longer (and the G_2 cyclins peak later) than they do during yeast growth (Fig. 7) (20, 22, 79, 145). This delay is even more dramatic, given that the conditions used for hyphal induction routinely include a shift to a higher temperature, 37°C, which elevates basal rates of metabolism (20, 79, 145). The time of peak Ccn1 expression is affected by the type of hypha-inducing media used (20, 79, 145), pointing to the idea that many environmental and/or nutritional factors

TABLE 2 Cell cycle-related gene sequences in *C. albicans*

S. cerevisiae gene name(s)	*C. albicans* homolog (name)	Function or process monitored (in the case of checkpoints)	Gene description(s) for *C. albicans* or for *S. cerevisiae*[a]
Cyclin and CDK genes			
CDC28/CDK1	orf19.3856 (*CDC28*)	CDK	**Cyclin-dependent protein kinase; interacts with regulatory cyclins; involved in determination of cell morphology during the cell cycle; phosphorylated primarily by Swe1p, and phosphorylation is regulated by Hsl1p**
CLN1/CLN2	orf19.3207 (*CCN1*)	G_1 cyclin	**Required for hyphal growth maintenance (not initiation); cell cycle-regulated transcription (G_1/S); Cdc28p-Ccn1p initiates Cdc11p S394 phosphorylation on hyphal induction; expression in *S. cerevisiae* inhibits pheromone response**
	orf19.6028 (*HGC1*)	G_1 cyclin	**Hypha-specific cyclin-related protein involved in regulation of hyphal morphogenesis; Cdc28p-Hgc1p maintains Cdc11p S394 phosphorylation during hyphal growth**
CLN3	orf19.1960 (*CLN3*)	G_1 cyclin	**Depletion abolishes budding and causes hyphal growth defects; essential for yeast form growth**
CLB1,2	orf19.1446 (*CLB2*)	Mitotic cyclin	**Essential; required for wild-type mitotic exit; role in cell polarization; interacts with catalytic subunit Cdk1p**
CLB3,4	orf19.7186 (*CLB4*)	Mitotic cyclin	**Nonessential; negative regulator of pseudohyphal growth; dispensable for mitotic exit, cytokinesis**
CLB5,6	None	Mitotic cyclin	Involved in DNA replication during S phase; activates Cdc28p to promote initiation of DNA synthesis
PHO85	orf19.6846 (*PHO85*)	CDK	**Functional homolog of *S. cerevisiae* Pho85p, which is a CDK that regulates processes, including transcription of PHO genes involved in phosphate metabolism**
PCL1	orf19.2649 (*PCL1*)	Cyclin	**Cyclin homolog; expression induced upon filamentous growth and in response to alpha pheromone**
PCL2	orf19.402 (*PCL2*)	Cyclin	**Cyclin homolog; peak transcripton levels at G_1/S phase**
PCL6	orf19.4012 (*PCL5*)	Cyclin	**Protein similar to *S. cerevisiae PCL5* and other Pho85 cyclins; phosphorylates Gcn4, leading to its degradation**
PCL7	orf19.6225 (*PCL7*)	Cyclin	**Protein described as cyclin-like, possible Pho85p cyclin; downregulated under hyphal conditions**
DNA damage/replication checkpoint genes			
BRE1	orf19.976	DNA damage	E3 ubiquitin ligase; forms heterodimer with Rad6p to monoubiquinate histone H2B-K123, which is required for the subsequent methylation of histones H3-K4 and H3-K79; required for DSBR,[c] transcription, silencing, and checkpoint control
CHK1	None	DNA damage	Serine/threonine kinase and DNA damage checkpoint effector, mediates cell cycle arrest via phosphorylation of Pds1p; phosphorylated by checkpoint signal transducer Mec1p; homolog of *S. pombe* and mammalian Chk1 checkpoint kinase
CTF18	orf19.3239	DNA damage	Subunit of a complex with Ctf8p that shares some subunits with replication factor C and is required for sister chromatid cohesion; may have overlapping functions with Rad24p in the DNA damage replication checkpoint
DDI1	orf19.7258	DNA damage, replication	DNA damage-inducible v-SNARE binding protein; contains a ubiquitin-associated domain; may act as a negative regulator of constitutive exocytosis; may play a role in S-phase checkpoint control

(Continued on next page)

TABLE 2 Cell cycle-related gene sequences in *C. albicans* (*Continued*)

S. cerevisiae gene name(s)	*C. albicans* homolog (name)	Function or process monitored (in the case of checkpoints)	Gene description(s) for *C. albicans* or for *S. cerevisiae*[a]
DOT1	None	DNA damage[b]	Nucleosomal histone H3-Lys79 methylase; methylation is required for telomeric silencing, meiotic checkpoint control, and DNA damage response
DPB11	orf19.1434	Replication	Replication initiation protein that loads DNA pol epsilon onto prereplication complexes at origins; checkpoint sensor recruited to stalled replication forks by the checkpoint clamp complex, where it activates Mec1p; ortholog of human TopBP1
DUN1	orf19.4002	DNA damage	Cell cycle checkpoint serine/threonine kinase required for DNA damage-induced transcription of certain target genes, phosphorylation of Rad55p and Sml1p, and transient G_2/M arrest after DNA damage; also regulates postreplicative DNA repair
ESC2	orf19.2326	DNA damage	Protein involved in silencing; may recruit or stabilize Sir proteins; role in Rad51-dependent homologous recombination repair and intra S-phase DNA damage checkpoint; member of the RENi (Rad60-Esc2-Nip45) family of SUMO-like domain proteins
GCN2	orf19.6913	DNA damage	Protein kinase; phosphorylates the alpha-subunit of translation initiation factor eIF2 (Sui2p) in response to starvation; activated by uncharged tRNAs and the Gcn1p-Gcn20p complex; contributes to DNA damage checkpoint control
HUG1	None	DNA damage, replication	Protein involved in the Mec1p-mediated checkpoint pathway that responds to DNA damage or replication arrest; transcription is induced by DNA damage
IES4	None	Replication	Component of the INO80 chromatin remodeling complex and target of the Mec1p/Tel1p DNA damage signaling pathway; proposed to link chromatin remodeling to replication checkpoint responses
LCD1	None	DNA damage, replication	Essential protein required for the DNA integrity checkpoint pathways; interacts physically with Mec1p; putative homolog of *S. pombe* Rad26 and human ATRIP
MEC1	orf19.1283	DNA damage, replication	Genome integrity checkpoint protein and phosphatidylinositol kinase superfamily member; signal transducer required for cell cycle arrest and transcriptional responses prompted by damaged or unreplicated DNA; monitors and participates in meiotic recombination
MEC3	orf19.5485	DNA damage[b]	DNA damage and meiotic pachytene checkpoint protein; subunit of a heterotrimeric complex (Rad17p-Mec3p-Ddc1p) that forms a sliding clamp, loaded onto partial duplex DNA by a clamp loader complex; homolog of human and *S. pombe* Hus1
MEK1	orf19.1874	DNA damage[b]	Meiosis-specific serine/threonine protein kinase; functions in meiotic checkpoint; promotes recombination between homologous chromosomes by suppressing DSBR between sister chromatids
MRC1	orf19.658	Replication	S-phase checkpoint protein required for DNA replication; interacts with and stabilizes Pol2p at stalled replication forks during stress, where it forms a pausing complex with Tof1p and is phosphorylated by Mec1p; protects uncapped telomeres
RAD17	orf19.366	DNA damage[b]	Checkpoint protein involved in the activation of the DNA damage and meiotic pachytene checkpoints; with Mec3p and Ddc1p, forms a clamp that is loaded onto partial duplex DNA; homolog of human and *S. pombe* Rad1 and *Ustilago maydis* Rec1 proteins
RAD24	orf19.2728	DNA damage[b]	Checkpoint protein, involved in the activation of the DNA damage and meiotic pachytene checkpoints; subunit of a clamp loader that loads Rad17p-Mec3p-Ddc1p onto DNA; homolog of human and *S. pombe* Rad17 protein

RAD6	orf19.7195	DNA damage	Ubiquitin-conjugating enzyme (E2) involved in postreplication repair (as a heterodimer with Rad18p), DSBR and checkpoint control (as a heterodimer with Bre1p), ubiquitin-mediated N-end rule protein degradation (as a heterodimer with Ubr1p)
RAD53	orf19.6936	DNA damage[b]	Protein involved in regulation of DNA damage-induced filamentous growth; putative component of cell cycle checkpoint; ortholog of *S. cerevisiae* Rad53p, protein kinase required for cell cycle arrest in response to DNA damage
RAD9	None	DNA damage[b]	DNA damage-dependent checkpoint protein, required for cell cycle arrest in G_1/S, intra-S, and G_2/M; transmits checkpoint signal by activating Rad53p and Chk1p; hyperphosphorylated by Mec1p and Tel1p; potential Cdc28p substrate
RFX1	orf19.3865	DNA damage, replication	Major transcriptional repressor of DNA damage-regulated genes, recruits repressors Tup1p and Cyc8p to their promoters; involved in DNA damage and replication checkpoint pathway; similar to a family of mammalian DNA binding proteins (RFX1-RFX4)
RNR1/RNR3	orf19.5779	DNA damage, replication	One of two large regulatory subunits of ribonucleotide-diphosphate reductase; the RNR complex catalyzes rate-limiting step in dNTP[d] synthesis, regulated by DNA replication and DNA damage checkpoint pathways via localization of small subunits
RNR2/RNR4	orf19.5801	DNA damage, replication	(RNR[e]), small subunit; the RNR complex catalyzes the rate-limiting step in dNTP[c] synthesis and is regulated by DNA replication and DNA damage checkpoint pathways via localization of the small subunits
TEL1	orf19.5580	DNA damage	Protein kinase primarily involved in telomere length regulation; contributes to cell cycle checkpoint control in response to DNA damage; functionally redundant with Mec1p; homolog of human ataxia telangiectasia (ATM) gene
TOF1	orf19.4136	DNA damage, replication	Subunit of a replication-pausing checkpoint complex (Tof1p-Mrc1p-Csm3p) that acts at the stalled replication fork to promote sister chromatid cohesion after DNA damage, facilitating gap repair of damaged DNA; interacts with the MCM helicase
XRS2	None	DNA damage, replication	Protein required for DNA repair; component of the Mre11 complex, which is involved in double strand breaks, meiotic recombination, telomere maintenance, and checkpoint signaling
Spindle/ SAC[f] genes			
AME1	None	Spindle	Essential kinetochore protein associated with microtubules and spindle pole bodies; component of the kinetochore subcomplex COMA (Ctf19p, Okp1p, Mcm21p, Ame1p); involved in spindle checkpoint maintenance
BIR1	None	SAC	Subunit of chromosomal passenger complex (Ipl1p-Sli15p-Bir1p-Nbl1p), which regulates chromosome segregation; required for chromosome bi-orientation and for SAC activation upon reduced sister kinetochore tension
BUB1	orf19.2678	Spindle	Protein kinase that forms a complex with Mad1p and Bub3p that is crucial in the checkpoint mechanism required to prevent cell cycle progression into anaphase in the presence of spindle damage; associates with centromere DNA via Skp1p
BUB3	orf19.2655	Spindle	Kinetochore checkpoint WD40 repeat protein that localizes to kinetochores during prophase and metaphase; delays anaphase in the presence of unattached kinetochores; forms complexes with Mad1p-Bub1p and with Cdc20p, binds Mad2p and Mad3p
EOS1	orf19.6416	Spindle	Protein involved in N glycosylation; deletion mutation confers sensitivity to exidative stress and shows synthetic lethality with mutations in the spindle checkpoint genes BUB3 and MAD1; YNL080C is not an essential gene

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TABLE 2 Cell cycle-related gene sequences in *C. albicans (Continued)*

S. cerevisiae gene name(s)	*C. albicans* homolog (name)	Function or process monitored (in the case of checkpoints)	Gene description(s) for *C. albicans* or for *S. cerevisiae*[a]
IBD2	None	Spindle	Component of the BUB2-dependent spindle checkpoint pathway; interacts with Bfa1p and functions upstream of Bub2p and Bfa1p
KIN4	orf19.3751	Spindle	Serine/threonine protein kinase that inhibits the mitotic exit network when the spindle position checkpoint is activated; localized asymmetrically to mother cell cortex, spindle pole body, and bud neck
MAD1	orf19.6357	SAC	Coiled-coil protein involved in the SAC; phosphorylated by Mps1p upon checkpoint activation, which leads to inhibition of the activity of the anaphase promoting complex; forms a complex with Mad2p
MAD2	orf19.1040	SAC	Component of the SAC complex, which delays the onset of anaphase in cells with defects in mitotic spindle assembly; forms a complex with Mad1p
MAD3	None	SAC	Subunit of the SAC complex, which delays anaphase onset in cells with defects in mitotic spindle assembly; pseudosubstrate inhibitor of APC (Cdc20), the anaphase-promoting complex involved in securin (Pds1p) turnover
MPS1	None	Spindle	Dual-specificity kinase required for spindle pole body duplication and spindle checkpoint function; substrates include SPB proteins Spc42p, Spc110p, and Spc98p, mitotic exit network protein Mob1p, and checkpoint protein Mad1p
NUF2/SPC24 /SPC25	orf19.1941	Spindle	Component of the evolutionarily conserved kinetochore-associated Ndc80 complex (Ndc80p-Nuf2p-Spc24p-Spc25p); involved in chromosome segregation, spindle checkpoint activity, and kinetochore clustering
NUP53	None	Spindle	Subunit of the nuclear pore complex, interacts with karyopherin Kap121p or with Nup170p via overlapping regions of Nup53p; involved in activation of the spindle checkpoint mediated by the Mad1p-Mad2p complex
SGO1	orf19.3550	Spindle	Component of the spindle checkpoint; involved in sensing lack of tension on mitotic chromosomes; protects centromeric Rec8p at meiosis I; required for accurate chromosomal segregation at meiosis II and for mitotic chromosome stability
SLI15	orf19.6049	Spindle	Subunit of the conserved chromosomal passenger complex (CPC; Ipl1p-Sli15p-Bir1p-Nbl1p), which regulates kinetochore-microtubule attachments, activation of the spindle tension checkpoint, and mitotic spindle disassembly
TID3	orf19.2827	Spindle	Component of the evolutionarily conserved kinetochore-associated Ndc80 complex (Ndc80p-Nuf2p-Spc24p-Spc25p); conserved coiled-coil protein involved in chromosome segregation, spindle checkpoint activity, kinetochore assembly, and clustering
Morphogenesis/septin checkpoint genes			
HSL1	None	Morphogenesis	Nim1p-related protein kinase that regulates the morphogenesis and septin checkpoints; associates with the assembled septin filament; required along with Hsl7p for bud neck recruitment, phosphorylation, and degradation of Swe1p
KCC4	None	Morphogenesis	Protein kinase of the bud neck involved in the septin checkpoint; associates with septin proteins; negatively regulates Swe1p by phosphorylation; shows structural homology to bud neck kinases Gin4p and Hsl1p
SWE1	orf19.4867	Morphogenesis	Protein kinase that regulates the G_2/M transition by inhibition of Cdc28p kinase activity; localizes to the nucleus and to the daughter side of the mother-bud neck; homolog of *S. pombe* Wee1p; potential Cdc28p

Mitotic exit checkpoint genes			
AMN1	orf19.9083	Mitotic exit	Protein required for daughter cell separation, multiple mitotic checkpoints, and chromosome stability; contains 12 degenerate leucine-rich repeat motifs; expression is induced by the mitotic exit network
BFA1	orf19.6080	Mitotic exit	Component of the GTPase-activating Bfa1p-Bub2p complex involved in multiple cell cycle checkpoint pathways that control exit from mitosis
BUB2	orf19.5827	Mitotic exit	Mutation confers hypersensitivity to amphotericin B; induced upon adherence to polystyrene; fungus specific (no human or murine homolog)
Checkpoint inactivation			
PTC2	None	DNA checkpoint inactivation	Type 2C protein phosphatase; dephosphorylates Hog1p to limit maximal osmostress-induced kinase activity; dephosphorylates Ire1p to downregulate the unfolded protein response; dephosphorylates Cdc28p; role in DNA checkpoint inactivation
PTC3	orf19.2538	DNA checkpoint inactivation	Type 2C protein phosphatase; dephosphorylates Hog1p (see also Ptc2p) to limit maximal kinase activity induced by osmotic stress; dephosphorylates T169-phosphorylated Cdc28p (see also Ptc2p); role in DNA checkpoint inactivation
ULP2	None	Recovery from DNA damage and replication checkpoints	Peptidase that deconjugates Smt3/SUMO-1 peptides from proteins; plays a role in chromosome cohesion at centromeric regions and recovery from checkpoint arrest induced by DNA damage or DNA replication defects; potential Cdc28p substrate
Meiosis-specific checkpoints			
DDC1	orf19.245	Meiosis-pachytene	DNA damage checkpoint protein, part of a PCNA-like complex required for DNA damage response, required for pachytene checkpoint to inhibit cell cycle in response to unrepaired recombination intermediates; potential Cdc28p substrate
PCH2	None	Meiosis-pachytene	Nucleolar component of the pachytene checkpoint, which prevents chromosome segregation when recombination and chromosome synapsis are defective; also represses meiotic interhomolog recombination in the ribosomal DNA
SUM1	None	Meiosis-pachytene	Transcriptional repressor required for mitotic repression of middle sporulation-specific genes; also acts as general replication initiation factor; involved in telomere maintenance, chromatin silencing; regulated by pachytene checkpoint

[a]Descriptions for *C. albicans* genes are from the *Candida* Genome Database (http://www.candidagenome.org) and are in bold font; those for *S. cerevisiae* genes are from the *Saccharomyces* Genome Database (http://www.yeastgenome.org) and are in plain font.
[b]Involved in meiotic checkpoints as well.
[c]DSBR, double-strand break repair.
[d]dNTP, deoxynucleoside triphosphate.
[e]RNR, ribonucleotide-diphosphate reductase.
[f]SAC, spindle assembly checkpoint.

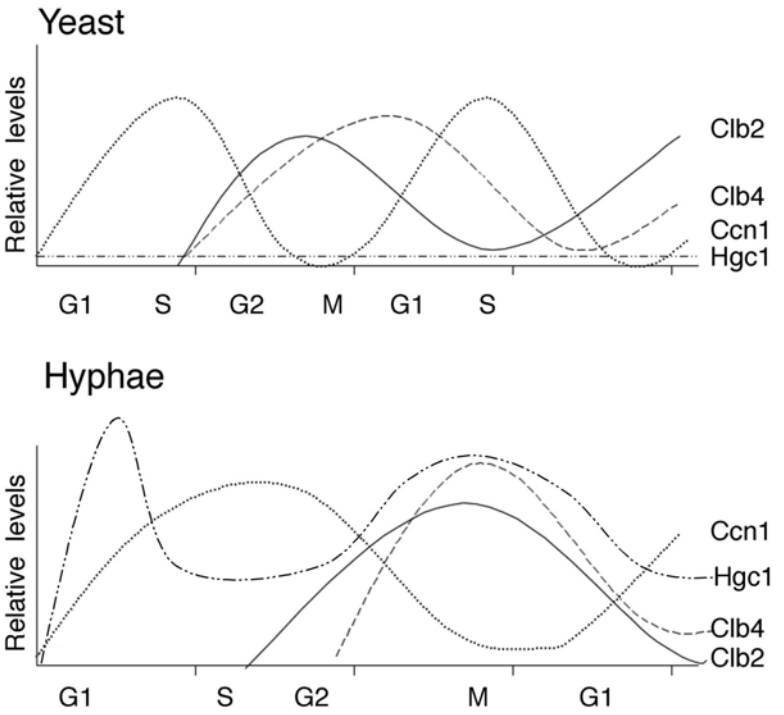

FIGURE 7 Cell cycle progression and cyclin levels differ in yeast cells and hyphae. In general, G_1 cyclins persist longer and mitotic cyclins appear later in hyphae than in yeast cells. Adapted from data in references 20, 22, 79, and 136. 10.1128/9781555817176.ch8f7

impinge on the dynamics of cell cycle progression and, in doing so, on the amount of time that cells undergo polarized growth. Furthermore, the extended presence of Ccn1/Cdc28 likely promotes a longer period of polarized growth, although it cannot explain the constitutive polarized growth seen in true hyphae. Strains lacking Ccn1 initiate germ tubes but then stop growing (79), demonstrating that Ccn1 is critically required for the maintenance of hyphal growth. In contrast, strains lacking Cln3 form hyphal filaments in response to serum, although nuclear migration into the hypha is delayed and the length of time between septations is increased despite similar hyphal lengths. This indicates that in hyphae, Cln3 regulates the timing of cell cycle events rather than the ability to execute them (9, 31).

Mitotic Cyclins

The G_2 cyclins, Clb2 and Clb4, increase in S/G_2 phase and peak in M phase in *C. albicans*, as they do in *S. cerevisiae* (Fig. 7) (20). Clb2 is essential for growth while Clb4 is not, suggesting that, as in *S. cerevisiae*, Clb2 is the major mitotic cyclin responsible for both nuclear and spindle functions in G_2/M as well as for the morphological changes that accompany cell cycle progression. G_2 cyclins are required for the apical-to-isotropic switch in growth: yeast cells lacking Clb2 continue to elongate without completing a cell cycle; those lacking Clb4 continue to grow but form elongated pseudohyphal cells (20). Thus, B cyclins are inhibitors of polarized growth and when their levels are affected, yeast cells become more polarized.

The peaks of mitotic cyclin levels are delayed, but the dynamics of their cycling are not fundamentally different in hyphal cells relative to yeast cells: expression levels peak at G_2/M (nuclear migration and division across the septin ring), which correlates with a trough in Ccn1 levels (Fig. 7) (20, 22). While early cell cycles in serum-induced hyphae appear to be similar to those of yeast cells (79), the timing of events is thought to be more divergent in later hyphal cell cycles (74).

In contrast to the situation in yeast cells, Clb4, rather than Clb2, is the primary mitotic cyclin in hyphal cells. The finding that Clb4 is important for hyphal morphogenesis is unexpected because during hyphal morphogenesis, no switch to isotropic growth occurs at G_2/M. In strains depleted for Clb2, hyphal morphogenesis continues although cell cycle events become severely delayed in mitosis, with the majority of cells containing highly extended spindles at later time points (20). In contrast, strains lacking or depleted for Clb4 form pseudohyphae in response to hyphal induction conditions (20); these pseudohyphae remain viable, although they also are delayed in their transit through M phase. In addition, strains lacking Fkh2, a transcription factor necessary for the induction of Clb4 and other genes important for S/G_2 phase (21, 33), grow with a pseudohyphal morphology under hyphal induction conditions. Thus, *C. albicans* mutants that affect G_2 phase are less polarized during hyphal growth, suggesting that despite the role of these factors as negative regulators of polarized growth in yeast cells, they must use a different, as-yet-uncharacterized mechanism to ensure the constitutive maintenance of polarized growth in hyphal cells. One possibility is that the mitotic cyclins regulate the hypha-repressing transcription factors Tup1 and Nrg1. Deletion of these genes, like deletion of Clb4, results in pseudohyphal growth under hyphal growth conditions (25, 26). Thus, Tup1 and Nrg1 have a

positive role in hyphal morphogenesis in addition to a hypha-repressing role in response to yeast form conditions. While no epistasis experiments have been done, it is tempting to speculate that in hypha-inducing media, Tup1 and Nrg1 regulate cell cycle dynamics to promote hyphal over pseudohyphal growth.

Regulation of the Hypha-Specific Cyclin Hgc1 and Its Downstream Effectors

Hgc1 is most similar in primary sequence to G_1 cyclins such as *S. cerevisiae* Cln1 and Cln2 (145). Hgc1 levels oscillate during the cell cycle, albeit differently than Ccn1 (136). *HGC1* expression is rapidly induced after transfer of yeast cells to hypha-inducing media and then falls when the *CCN1* transcript levels rise around the G_1/S transition. Similar to Ccn1, Hgc1 is not required for initial germ tube emergence but is required for germ tube elongation in all hypha-inducing media tested (136, 146). In addition, *hgc1* null strains have defects in the maintenance of cell-cell attachments during hyphal morphogenesis, consistent with the role of Hgc1 in directing the formation of hypha-specific cell biological features. Later, *HGC1* expression peaks around the G_2/M phase and then decreases again at the subsequent G_1/S transition (Fig. 7) (136). Hgc1 exhibits enhanced expression in the apical, growing hyphal cell, which is facilitated by the localization of *HGC1* transcript to the apical cell (136). While the mechanism of *HGC1* transcript localization is not known, it seems reasonable that it will involve RNA trafficking mechanisms like the She3 system that localizes transcripts to hyphal tips (40). For example, the mRNA for the secreted aspartyl proteinase Sap5 is transported to hyphal tips in a She3-dependent mechanism (40). Deletion studies show that Hgc1 is required for germ tube elongation in all hypha-inducing media tested (136, 145). Ectopic expression of Hgc1, however, is not sufficient for the induction of hyphal growth, suggesting that Hgc1 regulates only one of multiple coordinated processes required for the formation of hyphae. This is consistent with the large number of genes and gene products that are regulated differentially in hyphae relative to yeast cells (95) and that generate morphogenesis defects when present in only one, rather than two, copies (128).

Hgc1 is the only hypha-specific cyclin, and therefore, as expected, it makes important contributions to the unique cell biological features of hyphal cells. In particular, recent data show that Hgc1 is required for two hyphal characteristics: (i) constitutive polarization of growth in the face of continued mitotic cell cycles in apical cells and (ii) repression of cell separation at cytokinesis during hyphal morphogenesis.

One way that Hgc1 supports constitutive polarization of growth is by negative regulation of Rga2, a GAP of the major morphogenesis Rho-type GTPase Cdc42 (Fig. 8) (146). Hgc1 phosphorylates Rga2, thereby preventing it from localizing to hyphal tips (146) and from deactivating Cdc42. Similarly, loss of the Cdc42 GAPs (Bem3 or Rga2) is sufficient to induce hyphal morphogenesis (in media that promote pseudohyphal growth) (35). Thus, enhancement of the amount of active GTP-bound Cdc42, either by deletion of Cdc42 GAPs or by activation of Hgc1 with its concomitant effect on Rga2 localization, is sufficient to drive hyphal-form growth.

The second way that Hgc1 supports continued polarized growth in hyphae is by regulation of the Cdc11 septin. Septins localize to hyphal tips in addition to the site of future cytokinesis (44, 138), and Hgc1 likely acts on Cdc11 in the hyphal tips to promote constitutive polarized growth. Ccn1/Cdc28 and Hgc1/Cdc28 sequentially phosphorylate Cdc11, which is necessary for the maintenance of hyphal growth (112). Upon hyphal induction, Cdc11 is initially phosphorylated at Ser395 by Ccn1/Cdc28, and this phosphorylation state is then maintained by Hgc1/Cdc28 during hyphal elongation (112). Consistent with a role for Cdc11 in hyphal growth maintenance, deletion of *CDC11* causes defects in hyphal morphogenesis (138) and mislocalization of Sec3, a landmark protein of the exocyst, which is a complex of proteins that directs secretory vesicles to the site of growth at hyphal tips (76). Together, these observations suggest that Hgc1 achieves hyphal growth maintenance by activating Cdc11 at, and thereby targeting Sec3 to, hyphal tips. Thus, during normal hyphal morphogenesis, Hgc1 likely maintains constitutive polarization of growth in at least two ways: by inhibiting the activity of a Cdc42 GAP (Rga2) and by activating a tip-localized septin structure that targets secretion, and thereby growth, to the narrow zone of the hyphal tip (Fig. 8) (137).

Strains lacking Hgc1 are also unable to maintain cell-cell attachment after cytokinesis (145), pointing to a role for Hgc1 in cell separation, a process that distinguishes yeast from hyphal growth. Cell separation in yeast requires the recruitment of the Cdc14 phosphatase to the septin ring; accordingly, in hyphae, Hgc1 ensures that Cdc14 does not localize to the septin ring (32). Hgc1 phosphorylates Sep7, a septin protein that affects the increased exchange dynamics of septin protein Cdc10 between the ring and the cytoplasm and, in doing so, prevents Cdc14 recruitment to the cytokinetic ring (51). Indeed, septin ring dynamics are similar in hyphae lacking either Hgc1 or Sep7 (51), supporting the idea that Hgc1 and Sep7 act together to inhibit cell separation in hyphae.

Hgc1 has a second mechanistic role in keeping hyphal cells attached: it controls the transcription of genes encoding proteins required for septum degradation, a necessary step in cell separation. During *S. cerevisiae* yeast growth, septa are degraded by enzymes encoded by genes whose transcription is induced by Ace2. Ace2 is a transcription factor expressed during G_2/M that regulates the expression of M/G_1-phase-specific genes (33), including *CHT3*, which encodes a chitinase (71). During *C. albicans* hyphal growth, Hgc1/Cdc28 phosphorylates and activates Efg1, a transcription factor that negatively regulates Ace2 target genes, resulting in a cascade of events that inhibit septum degradation. Thus, Hgc1 prevents cell separation during normal hyphal growth in two ways: by decreasing septin ring affinity for Cdc14 and by negative transcriptional regulation of genes involved in septum degradation (Fig. 8).

Not surprisingly, Hgc1 expression is affected by signaling pathways that respond to hyphal induction conditions. Kadosh and Johnson used whole-genome DNA microarrays to identify approximately 60 hypha-specific genes that were induced in response to serum and 37°C. Of note, about half of them were negatively regulated by transcriptional repressors during yeast growth (69). In particular, serum and a temperature of 37°C downregulate the transcriptional corepressors Nrg1 and Tup1, effectively upregulating the transcriptional activator Ume6 (12, 69, 144), which positively regulates hyphal growth. Importantly, *HGC1* expression is partially dependent upon Ume6 (Fig. 8) (28, 144). In addition, Efg1 and Cph1/2 also regulate Hgc1 expression in a Ume6-dependent manner (144). Thus, relief from negative regulation is an important mechanism for activating the hyphal morphogenesis program.

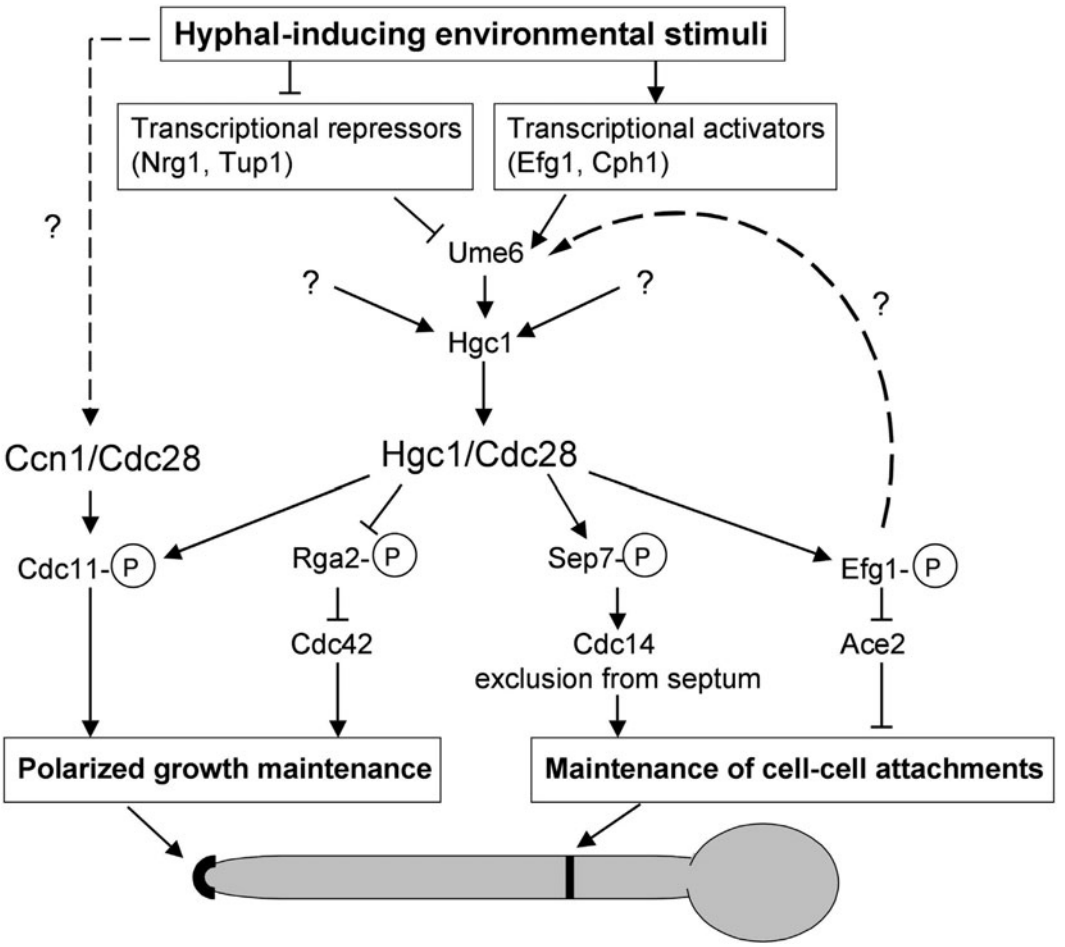

FIGURE 8 Model of G_1 cyclin-mediated maintenance of *C. albicans* hyphal growth and hypha-specific cellular features. Two cyclin-CDK complexes, Ccn1-Cdc28 and Hgc1-Cdc28, act sequentially to activate the Cdc11 septin and to promote polarized growth. In addition, Hgc1-Cdc28 phosphorylates, and inhibits the localization of, the Cdc42 GAP Rga2, which results in enhancement of Cdc42 activity at the hyphal tip. Hgc1-Cdc28 also promotes the maintenance of cell-cell attachments through two pathways: phosphorylation of Sep7 to prevent Cdc14 from acting at the septum and phosphorylation of Efg1 to inhibit the expression of Ace2-activated target genes. *HGC1* expression is partially dependent upon the hypha-specific transcriptional activator *UME6* (28, 144). Dotted arrows with question marks indicate possible interactions that have not been demonstrated conclusively. 10.1128/9781555817176.ch8f8

Pho85 Kinase and Pcl Cyclins

Pho85 is a CDK in *S. cerevisiae* that associates with a large number of Pcl cyclins (reviewed in reference 65). The *C. albicans* genome sequence predicts a Pho85 homolog (91) and four Pcl cyclins (numbered based on the closest *S. cerevisiae* homolog): Pcl1, Pcl2, Pcl5, and Pcl7 (Table 2). Of these, only *C. albicans* Pcl2 has been reported to have a potential role in cell cycle progression (9, 41), although the others have potential roles in morphogenesis. Pcl1 expression is induced under filamentous growth conditions (69), and Pcl7 expression is downregulated under hyphal conditions (57), although no phenotypic analysis has been performed to determine if these genes are important for morphogenesis or cell cycle progression. Pcl5 is involved in the filamentous growth response to amino acid starvation by virtue of its role in Gcn4 degradation (49). However, it is unclear if the observed morphogenesis defect in *pcl5* null mutants is due to a cell cycle perturbation.

Pcl2, however, appears to be a cell cycle regulator. Like Ccn1, Pcl2 expression peaks in G_1/S (33). If Cln3 is repressed, Pcl2 expression also drops even after the *cln3* mutants resume hyphal growth (9). In addition, *PCL2* is repressed during normal hyphal growth and increased in response to the hypha-inhibitory compound farnesol (41), consistent with the idea that Pcl2 may be a yeast cell cycle-specific factor. Mutations in *PCL2* would shed light on this issue.

STATIONARY PHASE AND FARNESOL

C. albicans cells enter quiescence, or stationary phase, after approximately 5 days of growth in rich medium. Accordingly,

the transcription levels of genes encoding CDKs (e.g., *CDC28*) and other kinases involved in cell cycle progression and genes involved in DNA replication, mitosis, and polarized growth decrease at this time (130). In contrast, in stationary phase there is increased expression of genes involved in gluconeogenesis, stress resistance, adherence, and DNA repair as well as genes associated with virulence and drug resistance (130). When diluted into fresh medium, cells exit stationary phase and there is a rapid induction of ribosomal protein and RNA production genes (41).

Importantly, release of cells from stationary phase is a potent, albeit transient, inducer of hyphal morphogenesis (41). As yeast cells move further into exponential phase, they gradually lose the ability to form hyphae. The mechanism responsible for this process involves the expression of inhibitory molecules, such as the quorum-sensing molecule farnesol, which increase in concentration as the culture reaches saturation (41, 63). Dilution of stationary-phase yeast cells into fresh medium dilutes these inhibitory molecules and thereby relieves the repression of hyphal growth. In addition, inclusion of farnesol in hypha-inducing medium inhibits expression of *HGC1* and *CLN3* relative to their expression levels in hypha-inducing medium without farnesol. Thus, production of quorum-sensing molecules and their effect on cell cycle regulators constitute a mechanism for influencing morphogenesis in *C. albicans*.

CELL CYCLE PERTURBATION AND MORPHOGENESIS

Recent reports have begun to provide insight into the mechanisms that mediate the induction of filamentous growth by cell cycle perturbations. Depletion of Cdc28, the major CDK, causes a range of elongated morphologies that have features of both pseudohyphae and true hyphae (129), under either yeast or hyphal growth conditions. This is likely due to perturbation of multiple cyclin-CDK activities, depending upon the cell cycle stage of each individual cell at the time that Cdc28 levels became limiting. Interestingly, Cdc28 repression also affects a number of transcription factors that regulate morphogenesis and/or the cell cycle (e.g., Nrg1, Efg1, and Fkh2) and, perhaps as a consequence, it also alters expression of hypha-specific genes (e.g., Hwp1, Rbt4, and Ece1) (129). Similarly, cells treated with the DNA synthesis inhibitor hydroxyurea (HU), cells depleted of Cdc5 (polo-like kinase important for spindle integrity), and cells lacking Rad52 (DNA repair and homologous recombination) express increased levels of hypha-specific genes (5, 8) and form hyperpolarized daughter cells. For HU-treated and *cdc5* null cells, the transcriptional response correlated with additional hyphal-like cellular features: lack of constrictions at septa and movement of the nucleus into the polarized daughter cell prior to division (8). Of note, while they cause defects at very different cell cycle stages, adenylate cyclase (Cdc35/Cyr1) was required (and Efg1 or Cph1 transcription factors were dispensable) for filamentous growth in response to all three perturbations (5, 8). This indicates that the cAMP/PKA pathway is a primary component of the mechanism that links cell cycle delays to filamentous growth. The dependence of the filamentous phenotype of *clb4*, *fkh2*, and *cln3* null cells on adenylate cyclase has not been studied. Of note, like the other perturbations, filamentous growth of *fkh2* mutants does not require Efg1 and Cph1 (21), while filamentous growth of *cln3* mutants and HU-induced filaments requires Ras1 (7, 9), an upstream signaling component of the cAMP/PKA pathway. Together, these observations are consistent with the idea that cell cycle perturbations from many causes converge on the cAMP/PKA pathway to execute a filamentous growth response to different types of cell cycle stress/delay/arrest.

In summary, it is clear that many mutants that affect cell cycle progression also induce a filamentous growth response at the transcriptional level and that this response resembles the response of wild-type, untreated cells to hypha-inducing conditions. Because hyphal growth is associated with virulence potential, it has been suggested that cell cycle perturbations induce filamentous growth to provide an "escape route" for yeast cells in response to stresses encountered in vivo (139). It seems reasonable that such a cell elongation mechanism may allow the yeast cell to move into a neighboring, more hospitable host niche and thus avoid host antifungal strategies.

CELL CYCLE CHECKPOINTS

In eukaryotic cells, checkpoints delay cell cycle progression to provide time for repair of defects and ensure the fidelity of cell division. Some cell biological features that are monitored by checkpoints include the accuracy of DNA replication (during S phase), the presence of DNA damage (at both G_1 and G_2 phases of the cell cycle), the assembly and alignment of the mitotic spindle (at M phase), and bud morphogenesis (during G_2/M phase). The *C. albicans* genome contains homologs of many of the checkpoint complex components that have been described for *S. cerevisiae* (Table 2).

A common outcome of activation of cell cycle checkpoints in yeast cells is hyperpolarized growth. In some cases, this may be due to pausing of the cell cycle during the polarized growth phase (G_1/S) and an inability to progress into the isotropic phase (G_2/M). Thus, it follows that the highly polarized nature of *C. albicans* hyphae may involve the activity of cell cycle checkpoints to keep the cell from switching to isotropic growth. In *S. cerevisiae*, one way that cell cycle delay or arrest is achieved is by regulation of Cdc28 activity via inhibitory phosphorylation by Swe1, the so-called "morphogenesis checkpoint" (75). In *C. albicans*, Cdc28 phosphorylation at Tyr19 cycles (phosphorylates and dephosphorylates) with similar timing in yeast and hyphal cells (60), making it unlikely that the elongated shape of a hypha is due to changes in Cdc28 activity. In addition, deletion of *SWE1* causes slightly rounder yeast cells but does not affect normal pseudohyphal and hyphal morphogenesis (140). Thus, although Swe1 mediates the filamentous growth response due to *rad52* deletion (see above) (5), its role appears to be a response to defects in cell cycle progression rather than a role in normal hyphal morphogenesis response. Similarly, in cells lacking the spindle assembly checkpoint component Mad2, hyphal morphogenesis occurs normally (10), indicating that a Mad2-mediated checkpoint is not involved in normal hyphal morphogenesis. Importantly, despite the lack of a role in the hyphal morphogenesis response in vitro, deletion of *SWE1* and *MAD2* attenuates virulence in mouse models of systemic candidiasis (10, 48). Thus, the ability to appropriately delay cell cycle progression through checkpoint activation during growth in vivo appears to be important for virulence, presumably by enabling the pathogen to survive environmental stresses present in the host.

In contrast to the observations with Swe1 and Mad2, deletion of *RAD53*, which is involved in monitoring DNA integrity (DNA damage checkpoint), results in reduced germ tube formation (110), supporting the idea that some cell cycle checkpoints are activated and involved in the formation of true hyphae. In addition, Rad53 has a direct role in the filamentous growth phenotype that occurs in response to genotoxic stresses (e.g., DNA damage and inhibition of DNA replication) that is independent of its role in cell cycle arrest. In strains expressing a Rad53 FHA-1 domain mutant, the cell elongation phenotype induced by genotoxic stress is inhibited without a concomitant effect on cell cycle arrest (110). Thus, Rad53 may have separable functions in cell cycle delay and polarized growth. It has been proposed, by analogy with *S. cerevisiae* (42, 114), that Rad53 may affect morphogenesis directly by regulating septin activity within the cytokinetic ring (75, 86, 137). Consistent with this, mutations of Hsl1 and Gin4, which reside in the septin ring, and in *S.cerevisiae* affect the activity of the Swe1 morphogenesis checkpoint kinase (81), also cause elongated-cell phenotypes in *C. albicans* (140). Furthermore, when *RAD52* is deleted, *SWE1* is required for filamentous cells to be formed (5). As discussed above, Hgc1 regulates some hypha-specific cellular features via modification of septins, both at the hyphal tip and at the cytokinetic ring. Collectively, these results implicate septin structures as key effectors for cell cycle regulation of morphogenesis. In addition, the filamentous phenotypes of *cdc5* mutants require Bub2, the spindle checkpoint kinase (7). As discussed above, *cdc5* filaments also require components of the cAMP/PKA pathway (8). Thus, data are accumulating in support of a model in which genetic defects or environmental stresses induce filamentous growth by activating cell cycle checkpoint proteins, which, in turn, act via the cAMP/PKA pathway to execute filamentous growth.

SUMMARY

The dramatic morphological diversity within *C. albicans* as a single species, as well as among *Candida* species in general, has long fascinated fungal biologists. Because of the importance of morphogenesis in the pathogenesis process, it is essential that we understand how cell cycle regulation affects cell shape at the molecular level. The pendulum has swung back to the idea that morphogenesis occurs along a continuum and that the final shape of the organism is likely driven by the extent to which signals interface with cell cycle regulators to activate common effectors. It remains to be shown, however, how the variety of cell shapes specifically affects the ability to cause disease. Recent work has provided insight into how cell cycle regulators are linked to effectors of polarized growth and more precise characterization of the cell biological markers associated with one morphological form or another. Future work should aim to correlate morphology with the ability to execute steps in the pathogenesis of niche-specific candidal infections.

We are grateful to Rebecca Pulver, Kendra Van Beusekom, Jennifer Norton, and Danielle Hausauer for microscopy expertise, Tamar Lahav for help with the preparation of Table 2, and Kelly Bouchonville for helpful suggestions on the manuscript. We thank the National Institutes of Health (R01AI 057440 to CG, R01 DE14666, R01AI0624273 and R01AI075096 to JB) the University Pediatrics Foundation, the United States-Israel Binational Science Foundation, and the Minnesota Partnership for Biotechnology and Medical Genomics for financial support of our research programs.

REFERENCES

1. **Akashi, T., T. Kanbe, and K. Tanaka.** 1994. The role of the cytoskeleton in the polarized growth of the germ tube in *Candida albicans*. *Microbiology* **140**(Pt. 2)**:**271–280.
2. **Akisada, T., K. Harada, M. Niimi, and A. Kamaguchi.** 1983. Production of contiguously arranged chlamydospores in *Candida albicans*. *J. Gen. Microbiol.* **129:**2327–2330.
3. **Alby, K., and R. J. Bennett.** 2009. Stress-induced phenotypic switching in *Candida albicans*. *Mol. Biol. Cell* **20:**3178–3191.
4. **Amberg, D. C., J. E. Zahner, J. W. Mulholland, J. R. Pringle, and D. Botstein.** 1997. Aip3p/Bud6p, a yeast actin-interacting protein that is involved in morphogenesis and the selection of bipolar budding sites. *Mol. Biol. Cell* **8:**729–753.
5. **Andaluz, E., T. Ciudad, J. Gomez-Raja, R. Calderone, and G. Larriba.** 2006. Rad52 depletion in *Candida albicans* triggers both the DNA-damage checkpoint and filamentation accompanied by but independent of expression of hypha-specific genes. *Mol. Microbiol.* **59:**1452–1472.
6. **Anderson, J. M., and D. R. Soll.** 1986. Differences in actin localization during bud and hypha formation in the yeast *Candida albicans*. *J. Gen. Microbiol.* **132:**2035–2047.
7. **Bachewich, C., A. Nantel, and M. Whiteway.** 2005. Cell cycle arrest during S or M phase generates polarized growth via distinct signals in *Candida albicans*. *Mol. Microbiol.* **57:**942–959.
8. **Bachewich, C., D. Y. Thomas, and M. Whiteway.** 2003. Depletion of a polo-like kinase in *Candida albicans* activates cyclase-dependent hyphal-like growth. *Mol. Biol. Cell* **14:**2163–2180.
9. **Bachewich, C., and M. Whiteway.** 2005. Cyclin Cln3p links G1 progression to hyphal and pseudohyphal development in *Candida albicans*. *Eukaryot. Cell* **4:**95–102.
10. **Bai, C., N. Ramanan, Y. M. Wang, and Y. Wang.** 2002. Spindle assembly checkpoint component CaMad2p is indispensable for *Candida albicans* survival and virulence in mice. *Mol. Microbiol.* **45:**31–44.
11. **Bakerspigel, A., and S. Burke.** 1974. A possible function of the chlamydospores of *Candida albicans*. *Mycopathol. Mycol. Appl.* **54:**147–152.
12. **Banerjee, M., D. S. Thompson, A. Lazzell, P. L. Carlisle, C. Pierce, C. Monteagudo, J. L. Lopez-Ribot, and D. Kadosh.** 2008. *UME6*, a novel filament-specific regulator of *Candida albicans* hyphal extension and virulence. *Mol. Biol. Cell* **19:**1354–1365.
13. **Barelle, C. J., E. A. Bohula, S. J. Kron, D. Wessels, D. R. Soll, A. Schafer, A. J. Brown, and N. A. Gow.** 2003. Asynchronous cell cycle and asymmetric vacuolar inheritance in true hyphae of *Candida albicans*. *Eukaryot. Cell* **2:**398–410.
14. **Barelle, C. J., M. L. Richard, C. Gaillardin, N. A. Gow, and A. J. Brown.** 2006. *Candida albicans VAC8* is required for vacuolar inheritance and normal hyphal branching. *Eukaryot. Cell* **5:**359–367.
15. **Bartnicki-Garcia, S., D. D. Bartnicki, G. Gierz, R. Lopez-Franco, and C. E. Bracker.** 1995. Evidence that Spitzenkörper behavior determines the shape of a fungal hypha: a test of the hyphoid model. *Exp. Mycol.* **19:** 153–159.
16. **Barton, R., and K. Gull.** 1988. Variation in cytoplasmic microtubule organization and spindle length between the two forms of the dimorphic fungus *Candida albicans*. *J. Cell Sci.* **91**(Pt 2)**:**211–220.
17. **Bassilana, M., J. Hopkins, and R. A. Arkowitz.** 2005. Regulation of the Cdc42/Cdc24 GTPase module during *Candida albicans* hyphal growth. *Eukaryot. Cell* **4:** 588–603.

18. **Bastidas, R. J., and J. Heitman.** 2009. Trimorphic stepping stones pave the way to fungal virulence. *Proc. Natl. Acad. Sci. USA* **106:**351–352.
19. **Bennett, R. J., and A. D. Johnson.** 2005. Mating in *Candida albicans* and the search for a sexual cycle. *Annu. Rev. Microbiol.* **59:**233–255.
20. **Bensen, E. S., A. Clemente-Blanco, K. R. Finley, J. Correa-Bordes, and J. Berman.** 2005. The mitotic cyclins Clb2p and Clb4p affect morphogenesis in *Candida albicans*. *Mol. Biol. Cell* **16:**3387–3400.
21. **Bensen, E. S., S. G. Filler, and J. Berman.** 2002. A forkhead transcription factor is important for true hyphal as well as yeast morphogenesis in *Candida albicans*. *Eukaryot. Cell* **1:**787–798.
22. **Berman, J.** 2006. Morphogenesis and cell cycle progression in *Candida albicans*. *Curr. Opin. Microbiol.* **9:**595–601.
23. **Brand, A., S. Shanks, V. M. Duncan, M. Yang, K. Mackenzie, and N. A. Gow.** 2007. Hyphal orientation of *Candida albicans* is regulated by a calcium-dependent mechanism. *Curr. Biol.* **17:**347–352.
24. **Brand, A., A. Vacharaksa, C. Bendel, J. Norton, P. Haynes, M. Henry-Stanley, C. Wells, K. Ross, N. A. Gow, and C. A. Gale.** 2008. An internal polarity landmark is important for externally induced hyphal behaviors in *Candida albicans*. *Eukaryot. Cell* **7:**712–720.
25. **Braun, B. R., and A. D. Johnson.** 1997. Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. *Science* **277:**105–109.
26. **Braun, B. R., D. Kadosh, and A. D. Johnson.** 2001. *NRG1*, a repressor of filamentous growth in *C. albicans*, is down-regulated during filament induction. *EMBO J.* **20:**4753–4761.
27. **Bruatto, M., and A. Delu.** 1993. Microscopical evidences for the presence of a nucleus in *Candida albicans* chlamydoconidia. *Mycopathologia* **122:**103–105.
28. **Carlisle, P. L., M. Banerjee, A. Lazzell, C. Monteagudo, J. L. Lopez-Ribot, and D. Kadosh.** 2009. Expression levels of a filament-specific transcriptional regulator are sufficient to determine *Candida albicans* morphology and virulence. *Proc. Natl. Acad. Sci. USA* **106:**599–604.
29. **Chaffin, W. L.** 1984. The relationship between yeast cell size and cell division in *Candida albicans*. *Can. J. Microbiol.* **30:**192–203.
30. **Chant, J., and I. Herskowitz.** 1991. Genetic control of bud site selection in yeast by a set of gene products that constitute a morphogenetic pathway. *Cell* **65:**1203–1212.
31. **Chapa y Lazo, B., S. Bates, and P. Sudbery.** 2005. The G1 cyclin Cln3 regulates morphogenesis in *Candida albicans*. *Eukaryot. Cell* **4:**90–94.
32. **Clemente-Blanco, A., A. Gonzalez-Novo, F. Machin, D. Caballero-Lima, L. Aragon, M. Sanchez, C. R. de Aldana, J. Jimenez, and J. Correa-Bordes.** 2006. The Cdc14p phosphatase affects late cell-cycle events and morphogenesis in *Candida albicans*. *J. Cell Sci.* **119:**1130–1143.
33. **Côte, P., H. Hogues, and M. Whiteway.** 2009. Transcriptional analysis of the *Candida albicans* cell cycle. *Mol. Biol. Cell* **20:**3363–3373.
34. **Côte, P., and M. Whiteway.** 2008. The role of *Candida albicans FAR1* in regulation of pheromone-mediated mating, gene expression and cell cycle arrest. *Mol. Microbiol.* **68:**392–404.
35. **Court, H., and P. Sudbery.** 2007. Regulation of Cdc42 GTPase activity in the formation of hyphae in *Candida albicans*. *Mol. Biol. Cell* **18:**265–281.
36. **Crampin, H., K. Finley, M. Gerami-Nejad, H. Court, C. Gale, J. Berman, and P. Sudbery.** 2005. *Candida albicans* hyphae have a Spitzenkörper that is distinct from the polarisome found in yeast and pseudohyphae. *J. Cell Sci.* **118:**2935–2947.
37. **Crombie, T., N. A. Gow, and G. W. Gooday.** 1990. Influence of applied electrical fields on yeast and hyphal growth of *Candida albicans*. *J. Gen. Microbiol.* **136:**311–317.
38. **Dunkler, A., A. Walther, C. A. Specht, and J. Wendland.** 2005. *Candida albicans CHT3* encodes the functional homolog of the Cts1 chitinase of *Saccharomyces cerevisiae*. *Fungal Genet. Biol.* **42:**935–947.
39. **Dunkler, A., and J. Wendland.** 2007. *Candida albicans* Rho-type GTPase-encoding genes required for polarized cell growth and cell separation. *Eukaryot. Cell* **6:**844–854.
40. **Elson, S. L., S. M. Noble, N. V. Solis, S. G. Filler, and A. D. Johnson.** 2009. An RNA transport system in *Candida albicans* regulates hyphal morphology and invasive growth. *PLoS Genet.* **5:**e1000664.
41. **Enjalbert, B., and M. Whiteway.** 2005. Release from quorum-sensing molecules triggers hyphal formation during *Candida albicans* resumption of growth. *Eukaryot. Cell* **4:**1203–1210.
42. **Enserink, J. M., M. B. Smolka, H. Zhou, and R. D. Kolodner.** 2006. Checkpoint proteins control morphogenetic events during DNA replication stress in *Saccharomyces cerevisiae*. *J. Cell Biol.* **175:**729–741.
43. **Filler, S. G., and D. C. Sheppard.** 2006. Fungal invasion of normally non-phagocytic host cells. *PLoS Pathog.* **2:**e129.
44. **Finley, K. R., and J. Berman.** 2005. Microtubules in *Candida albicans* hyphae drive nuclear dynamics and connect cell cycle progression to morphogenesis. *Eukaryot. Cell* **4:**1697–1711.
45. **Finley, K. R., K. J. Bouchonville, A. Quick, and J. Berman.** 2008. Dynein-dependent nuclear dynamics affect morphogenesis in *Candida albicans* by means of the Bub2p spindle checkpoint. *J. Cell Sci.* **121:**466–476.
46. **Futcher, B.** 1999. Cell cycle synchronization. *Methods Cell Sci.* **21:**79–86.
47. **Gale, C. A., M. Gerami-Nejad, M. McClellan, S. Vandoninck, M. S. Longtine, and J. Berman.** 2001. *Candida albicans* Int1p interacts with the septin ring in yeast and hyphal cells. *Mol. Biol. Cell* **12:**3538–3549.
48. **Gale, C. A., M. D. Leonard, K. R. Finley, L. Christensen, M. McClellan, D. Abbey, C. Kurischko, E. Bensen, I. Tzafrir, S. Kauffman, J. Becker, and J. Berman.** 2009. *SLA2* mutations cause *SWE1*-mediated cell cycle phenotypes in *Candida albicans* and *Saccharomyces cerevisiae*. *Microbiology* **155:**3847–3859.
49. **Gildor, T., R. Shemer, A. Atir-Lande, and D. Kornitzer.** 2005. Coevolution of cyclin Pcl5 and its substrate Gcn4. *Eukaryot. Cell* **4:**310–318.
50. **Girbardt, M.** 1957. Der Spitzenkörper von *Polystictus versicolor*. *Planta* **50:**47–59.
51. **Gonzalez-Novo, A., J. Correa-Bordes, L. Labrador, M. Sanchez, C. R. Vazquez de Aldana, and J. Jimenez.** 2008. Sep7 is essential to modify septin ring dynamics and inhibit cell separation during *Candida albicans* hyphal growth. *Mol. Biol. Cell* **19:**1509–1518.
52. **Gow, N. A.** 1997. Germ tube growth of *Candida albicans*. *Curr. Top. Med. Mycol.* **8:**43–55.
53. **Gow, N. A., and G. W. Gooday.** 1987. Cytological aspects of dimorphism in *Candida albicans*. *Crit. Rev. Microbiol.* **15:**73–78.
54. **Gow, N. A., and G. W. Gooday.** 1982. Growth kinetics and morphology of colonies of the filamentous form of *Candida albicans*. *J. Gen. Microbiol.* **128:**2187–2194.
55. **Gow, N. A., and G. W. Gooday.** 1982. Vacuolation, branch production and linear growth of germ tubes in *Candida albicans*. *J. Gen. Microbiol.* **128:**2195–2198.
56. **Guan, K. L., and Y. Rao.** 2003. Signalling mechanisms mediating neuronal responses to guidance cues. *Nat. Rev. Neurosci.* **4:**941–956.

57. **Harcus, D., A. Nantel, A. Marcil, T. Rigby, and M. Whiteway.** 2004. Transcription profiling of cyclic AMP signaling in *Candida albicans*. *Mol. Biol. Cell* **15:** 4490–4499.
58. **Hausauer, D. L., M. Gerami-Nejad, C. Kistler-Anderson, and C. A. Gale.** 2005. Hyphal guidance and invasive growth in *Candida albicans* require the Ras-like GTPase Rsr1p and its GTPase-activating protein Bud2p. *Eukaryot. Cell* **4:**1273–1286.
59. **Hazan, I., and H. Liu.** 2002. Hyphal tip-associated localization of Cdc42 is F-actin dependent in *Candida albicans*. *Eukaryot. Cell* **1:**856–864.
60. **Hazan, I., M. Sepulveda-Becerra, and H. Liu.** 2002. Hyphal elongation is regulated independently of cell cycle in *Candida albicans*. *Mol. Biol. Cell* **13:**134–145.
61. **Herrero, A. B., M. C. Lopez, L. Fernandez-Lago, and A. Dominguez.** 1999. *Candida albicans* and *Yarrowia lipolytica* as alternative models for analysing budding patterns and germ tube formation in dimorphic fungi. *Microbiology* **145:**2727–2737.
62. **Homann, O. R., J. Dea, S. M. Noble, and A. D. Johnson.** 2009. A phenotypic profile of the *Candida albicans* regulatory network. *PLoS Genet.* **5:**e1000783.
63. **Hornby, J. M., E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, and K. W. Nickerson.** 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol.* **67:**2982–2992.
64. **Howell, A. S., N. S. Savage, S. A. Johnson, I. Bose, A. W. Wagner, T. R. Zyla, H. F. Nijhout, M. C. Reed, A. B. Goryachev, and D. J. Lew.** 2009. Singularity in polarization: rewiring yeast cells to make two buds. *Cell* **139:**731–743.
65. **Huang, D., H. Friesen, and B. Andrews.** 2007. Pho85, a multifunctional cyclin-dependent protein kinase in budding yeast. *Mol. Microbiol.* **66:**303–314.
66. **Irazoqui, J. E., A. S. Howell, C. L. Theesfeld, and D. J. Lew.** 2005. Opposing roles for actin in Cdc42p polarization. *Mol. Biol. Cell* **16:**1296–1304.
67. **Jansons, V. K., and W. J. Nickerson.** 1970. Induction, morphogenesis, and germination of the chlamydospore of *Candida albicans*. *J. Bacteriol.* **104:**910–921.
68. **Jorgensen, P., and M. Tyers.** 2004. How cells coordinate growth and division. *Curr. Biol.* **14:**R1014–R1027.
69. **Kadosh, D., and A. D. Johnson.** 2005. Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: a genome-wide analysis. *Mol. Biol. Cell* **16:**2903–2912.
70. **Kaksonen, M., Y. Sun, and D. G. Drubin.** 2003. A pathway for association of receptors, adaptors, and actin during endocytic internalization. *Cell* **115:**475–487.
71. **Kelly, M. T., D. M. MacCallum, S. D. Clancy, F. C. Odds, A. J. Brown, and G. Butler.** 2004. The *Candida albicans* CaACE2 gene affects morphogenesis, adherence and virulence. *Mol. Microbiol.* **53:**969–983.
72. **Kim, S. M., D. D. Dubey, and J. A. Huberman.** 2003. Early-replicating heterochromatin. *Genes Dev.* **17:**330–335.
73. **Koren, A., H. J. Tsai, I. Tirosh, L. Burrack, N. Barkai, and J. Berman.** 2010. Epigenetically-inherited centromere and neocentromere DNA replicates earliest in S-phase. *PLOS Genet.* **6:**e1001068.
74. **Kron, S. J., and N. A. Gow.** 1995. Budding yeast morphogenesis: signalling, cytoskeleton and cell cycle. *Curr. Opin. Cell Biol.* **7:**845–855.
75. **Lew, D. J.** 2003. The morphogenesis checkpoint: how yeast cells watch their figures. *Curr. Opin. Cell Biol.* **15:**648–653.
76. **Li, C. R., R. T. Lee, Y. M. Wang, X. D. Zheng, and Y. Wang.** 2007. *Candida albicans* hyphal morphogenesis occurs in Sec3p-independent and Sec3p-dependent phases separated by septin ring formation. *J. Cell Sci.* **120:** 1898–1907.
77. **Li, C. R., Y. M. Wang, X. De Zheng, H. Y. Liang, J. C. Tang, and Y. Wang.** 2005. The formin family protein CaBni1p has a role in cell polarity control during both yeast and hyphal growth in *Candida albicans*. *J. Cell Sci.* **118:**2637–2648.
78. **Li, W. J., Y. M. Wang, X. D. Zheng, Q. M. Shi, T. T. Zhang, C. Bai, D. Li, J. L. Sang, and Y. Wang.** 2006. The F-box protein Grr1 regulates the stability of Ccn1, Cln3 and Hof1 and cell morphogenesis in *Candida albicans*. *Mol. Microbiol.* **62:**212–226.
79. **Loeb, J. D., M. Sepulveda-Becerra, I. Hazan, and H. Liu.** 1999. A G_1 cyclin is necessary for maintenance of filamentous growth in *Candida albicans*. *Mol. Cell. Biol.* **19:**4019–4027.
80. **Longtine, M. S., and E. Bi.** 2003. Regulation of septin organization and function in yeast. *Trends Cell Biol.* **13:**403–409.
81. **Longtine, M. S., C. L. Theesfeld, J. N. McMillan, E. Weaver, J. R. Pringle, and D. J. Lew.** 2000. Septin-dependent assembly of a cell cycle-regulatory module in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **20:**4049–4061.
82. **Lopez-Franco, R., S. Bartnicki-Garcia, and C. E. Bracker.** 1994. Pulsed growth of fungal hyphal tips. *Proc. Natl. Acad. Sci. USA* **91:**12228–12232.
83. **Luo, J., E. A. Vallen, C. Dravis, S. E. Tcheperegine, B. Drees, and E. Bi.** 2004. Identification and functional analysis of the essential and regulatory light chains of the only type II myosin Myo1p in *Saccharomyces cerevisiae*. *J. Cell Biol.* **165:**843–855.
84. **Martin, R., A. Walther, and J. Wendland.** 2005. Ras1-induced hyphal development in *Candida albicans* requires the formin Bni1. *Eukaryot. Cell* **4:**1712–1724.
85. **Martin, S. W., L. M. Douglas, and J. B. Konopka.** 2005. Cell cycle dynamics and quorum sensing in *Candida albicans* chlamydospores are distinct from budding and hyphal growth. *Eukaryot. Cell* **4:**1191–1202.
86. **McMillan, J. N., M. S. Longtine, R. A. Sia, C. L. Theesfeld, E. S. Bardes, J. R. Pringle, and D. J. Lew.** 1999. The morphogenesis checkpoint in *Saccharomyces cerevisiae*: cell cycle control of Swe1p degradation by Hsl1p and Hsl7p. *Mol. Cell. Biol.* **19:**6929–6939.
87. **Merson-Davies, L. A., and F. C. Odds.** 1989. A morphology index for characterization of cell shape in *Candida albicans*. *J. Gen. Microbiol.* **135:**3143–3152.
88. **Miller, M. G., and A. D. Johnson.** 2002. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell* **110:**293–302.
89. **Miller, S. E., and W. R. Finnerty.** 1979. Age-related physiological studies comparing *Candida albicans* chlamydospores to yeasts. *Can. J. Microbiol.* **25:**765–772.
90. **Mitchell, L. H., and D. R. Soll.** 1979. Commitment to germ tube or bud formation during release from stationary phase in *Candida albicans*. *Exp. Cell Res.* **120:**167–179.
91. **Miyakawa, Y.** 2000. Identification of a *Candida albicans* homologue of the *PHO85* gene, a negative regulator of the *PHO* system in *Saccharomyces cerevisiae*. *Yeast* **16:** 1045–1051.
92. **Montazeri, M., and H. G. Hedrick.** 1984. Factors affecting spore formation in a *Candida albicans* strain. *Appl. Environ. Microbiol.* **47:**1341–1342.
93. **Moran, G. P., D. M. MacCallum, M. J. Spiering, D. C. Coleman, and D. J. Sullivan.** 2007. Differential regulation of the transcriptional repressor *NRG1* accounts for altered host-cell interactions in *Candida albicans* and *Candida dubliniensis*. *Mol. Microbiol.* **66:**915–929.

94. **Moseley, J. B., and B. L. Goode.** 2006. The yeast actin cytoskeleton: from cellular function to biochemical mechanism. *Microbiol. Mol. Biol. Rev.* **70:**605–645.
95. **Nantel, A., D. Dignard, C. Bachewich, D. Harcus, A. Marcil, A. P. Bouin, C. W. Sensen, H. Hogues, M. van het Hoog, P. Gordon, T. Rigby, F. Benoit, D. C. Tessier, D. Y. Thomas, and M. Whiteway.** 2002. Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol. Biol. Cell* **13:**3452–3465.
96. **Nielsen, K., and J. Heitman.** 2007. Sex and virulence of human pathogenic fungi. *Adv. Genet.* **57:**143–173.
97. **Odds, F. C.** 1988. Candida *and Candidosis*, p. 42–59. Tindall, London, United Kingdom.
98. **Ozaki-Kuroda, K., Y. Yamamoto, H. Nohara, M. Kinoshita, T. Fujiwara, K. Irie, and Y. Takai.** 2001. Dynamic localization and function of Bni1p at the sites of directed growth in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **21:**827–839.
99. **Park, H. O., J. Chant, and I. Herskowitz.** 1993. *BUD2* encodes a GTPase-activating protein for Bud1/Rsr1 necessary for proper bud-site selection in yeast. *Nature* **365:**269–274.
100. **Pruyne, D., M. Evangelista, C. Yang, E. Bi, S. Zigmond, A. Bretscher, and C. Boone.** 2002. Role of formins in actin assembly: nucleation and barbed-end association. *Science* **297:**612–615.
101. **Rajnicek, A. M., L. E. Foubister, and C. D. McCaig.** 2006. Growth cone steering by a physiological electric field requires dynamic microtubules, microfilaments and Rac-mediated filopodial asymmetry. *J. Cell Sci.* **119:** 1736–1745.
102. **Rajnicek, A. M., L. E. Foubister, and C. D. McCaig.** 2006. Temporally and spatially coordinated roles for Rho, Rac, Cdc42 and their effectors in growth cone guidance by a physiological electric field. *J. Cell Sci.* **119:** 1723–1735.
103. **Raudonis, B. M., and A. G. Smith.** 1982. Germination of the chlamydospores of *Candida albicans*. *Mycopathologia* **78:**87–91.
104. **Reynaga-Pena, C. G., G. Gierz, and S. Bartnicki-Garcia.** 1997. Analysis of the role of the Spitzenkörper in fungal morphogenesis by computer simulation of apical branching in *Aspergillus niger*. *Proc. Natl. Acad. Sci. USA* **94:**9096–9101.
105. **Saville, S. P., A. L. Lazzell, A. K. Chaturvedi, C. Monteagudo, and J. L. Lopez-Ribot.** 2008. Use of a genetically engineered strain to evaluate the pathogenic potential of yeast cell and filamentous forms during *Candida albicans* systemic infection in immunodeficient mice. *Infect. Immun.* **76:**97–102.
106. **Sellam, A., C. Askew, E. Epp, F. Tebbji, A. Mullick, M. Whiteway, and A. Nantel.** 2010. Role of the transcription factor CaNdt80p in cell separation, hyphal growth and virulence in *Candida albicans*. *Eukaryot. Cell* **9:**634–644.
107. **Sevilla, M. J., and F. C. Odds.** 1986. Development of *Candida albicans* hyphae in different growth media—variations in growth rates, cell dimensions and timing of morphogenetic events. *J. Gen. Microbiol.* **132:** 3083–3088.
108. **Shannon, J. L.** 1981. Scanning and transmission electron microscopy of *Candida albicans* chlamydospores. *J. Gen. Microbiol.* **125:**199–203.
109. **Sheu, Y. J., B. Santos, N. Fortin, C. Costigan, and M. Snyder.** 1998. Spa2p interacts with cell polarity proteins and signaling components involved in yeast cell morphogenesis. *Mol. Cell. Biol.* **18:**4053–4069.
110. **Shi, Q. M., Y. M. Wang, X. D. Zheng, R. T. Lee, and Y. Wang.** 2007. Critical role of DNA checkpoints in mediating genotoxic-stress-induced filamentous growth in *Candida albicans*. *Mol. Biol. Cell* **18:**815–826.
111. **Shrewsbury, J. F. D.** 1934. The genus *Monilia*. *J. Pathol. Bacteriol.* **38:**313–354.
112. **Sinha, I., Y. M. Wang, R. Philp, C. R. Li, W. H. Yap, and Y. Wang.** 2007. Cyclin-dependent kinases control septin phosphorylation in *Candida albicans* hyphal development. *Dev. Cell* **13:**421–432.
113. **Slutsky, B., M. Staebell, J. Anderson, L. Risen, M. Pfaller, and D. R. Soll.** 1987. "White-opaque transition": a second high-frequency switching system in *Candida albicans*. *J. Bacteriol.* **169:**189–197.
114. **Smolka, M. B., S. H. Chen, P. S. Maddox, J. M. Enserink, C. P. Albuquerque, X. X. Wei, A. Desai, R. D. Kolodner, and H. Zhou.** 2006. An FHA domain-mediated protein interaction network of Rad53 reveals its role in polarized cell growth. *J. Cell Biol.* **175:** 743–753.
115. **Snyder, M.** 1989. The SPA2 protein of yeast localizes to sites of cell growth. *J. Cell Biol.* **108:**1419–1429.
116. **Soll, D. R.** 1997. Gene regulation during high-frequency switching in *Candida albicans*. *Microbiology* **143**(Pt. 2)**:** 279–288.
117. **Soll, D. R.** 2009. Why does *Candida albicans* switch? *FEMS Yeast Res.* **9:**973–989.
118. **Soll, D. R., and L. H. Mitchell.** 1983. Filament ring formation in the dimorphic yeast *Candida albicans*. *J. Cell Biol.* **96:**486–493.
119. **Soll, D. R., B. Morrow, and T. Srikantha.** 1993. High-frequency phenotypic switching in *Candida albicans*. *Trends Genet.* **9:**61–65.
120. **Song, Y., and J. Y. Kim.** 2006. Role of CaBud6p in the polarized growth of *Candida albicans*. *J. Microbiol.* **44:**311–319.
121. **Staib, P., and J. Morschhauser.** 2007. Chlamydospore formation in *Candida albicans* and *Candida dubliniensis*—an enigmatic developmental programme. *Mycoses* **50:**1–12.
122. **Staib, P., and J. Morschhauser.** 2005. Liquid growth conditions for abundant chlamydospore formation in *Candida dubliniensis*. *Mycoses* **48:**50–54.
123. **Stuart, D., and C. Wittenberg.** 1995. *CLN3*, not positive feedback, determines the timing of *CLN2* transcription in cycling cells. *Genes Dev.* **9:**2780–2794.
124. **Sudbery, P., N. Gow, and J. Berman.** 2004. The distinct morphogenic states of *Candida albicans*. *Trends Microbiol.* **12:**317–324.
125. **Sudbery, P. E.** 2001. The germ tubes of *Candida albicans* hyphae and pseudohyphae show different patterns of septin ring localization. *Mol. Microbiol.* **41:**19–31.
126. **Torosantucci, A., and A. Cassone.** 1983. Induction and morphogenesis of chlamydospores in an agerminative variant of *Candida albicans*. *Sabouraudia* **21:**49–57.
127. **Uchida, M., G. McDermott, M. Wetzler, M. A. Le Gros, M. Myllys, C. Knoechel, A. E. Barron, and C. A. Larabell.** 2009. Soft X-ray tomography of phenotypic switching and the cellular response to antifungal peptoids in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **106:** 19375–19380.
128. **Uhl, M. A., M. Biery, N. Craig, and A. D. Johnson.** 2003. Haploinsufficiency-based large-scale forward genetic analysis of filamentous growth in the diploid human fungal pathogen *C. albicans*. *EMBO J.* **22:**2668–2678.
129. **Umeyama, T., A. Kaneko, M. Niimi, and Y. Uehara.** 2006. Repression of *CDC28* reduces the expression of the morphology-related transcription factors, Efg1p, Nrg1p, Rbf1p, Rim101p, Fkh2p and Tec1p and induces cell elongation in *Candida albicans*. *Yeast* **23:**537–552.
130. **Uppuluri, P., and W. L. Chaffin.** 2007. Defining *Candida albicans* stationary phase by cellular and DNA replication,

gene expression and regulation. *Mol. Microbiol.* **64:**1572–1586.
131. **van der Walt, J. P.** 1967. Sexually active strains of *Candida albicans* and *Cryptococcus albidus*. *Antonie van Leeuwenhoek* **33:**246–256.
132. **Vernis, L., A. Abbas, M. Chasles, C. M. Gaillardin, C. Brun, J. A. Huberman, and P. Fournier.** 1997. An origin of replication and a centromere are both needed to establish a replicative plasmid in the yeast *Yarrowia lipolytica*. *Mol. Cell. Biol.* **17:**1995–2004.
133. **Veses, V., M. Casanova, A. Murgui, A. Dominguez, N. A. Gow, and J. P. Martinez.** 2005. *ABG1*, a novel and essential *Candida albicans* gene encoding a vacuolar protein involved in cytokinesis and hyphal branching. *Eukaryot. Cell* **4:**1088–1101.
134. **Veses, V., A. Richards, and N. A. Gow.** 2009. Vacuole inheritance regulates cell size and branching frequency of *Candida albicans* hyphae. *Mol. Microbiol.* **71:**505–519.
135. **Vidotto, V., M. Bruatto, G. Accattatis, and S. Caramello.** 1996. Observation on the nucleic acids in the chlamydospores of *Candida albicans*. *New Microbiol.* **19:**327–334.
136. **Wang, A., S. Lane, Z. Tian, A. Sharon, I. Hazan, and H. Liu.** 2007. Temporal and spatial control of *HGC1* expression results in Hgc1 localization to the apical cells of hyphae in *Candida albicans*. *Eukaryot. Cell* **6:**253–261.
137. **Wang, Y.** 2009. CDKs and the yeast-hyphal decision. *Curr. Opin. Microbiol.* **12:**644–649.
138. **Warenda, A. J., and J. B. Konopka.** 2002. Septin function in *Candida albicans* morphogenesis. *Mol. Biol. Cell* **13:**2732–2746.
139. **Whiteway, M., and C. Bachewich.** 2007. Morphogenesis in *Candida albicans*. *Annu. Rev. Microbiol.* **61:**529–553.
140. **Wightman, R., S. Bates, P. Amornrrattanapan, and P. Sudbery.** 2004. In *Candida albicans*, the Nim1 kinases Gin4 and Hsl1 negatively regulate pseudohypha formation and Gin4 also controls septin organization. *J. Cell Biol.* **164:**581–591.
141. **Wolfe, K. H., and D. C. Shields.** 1997. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387:**708–713.
142. **Yaar, L., M. Mevarech, and Y. Koltin.** 1997. A *Candida albicans RAS*-related gene (Ca*RSR1*) is involved in budding, cell morphogenesis and hypha development. *Microbiology* **143:**3033–3044.
143. **Yokoyama, K., H. Kaji, K. Nishimura, and M. Miyaji.** 1990. The role of microfilaments and microtubules in apical growth and dimorphism of *Candida albicans*. *J. Gen. Microbiol.* **136:**1067–1075.
144. **Zeidler, U., T. Lettner, C. Lassnig, M. Muller, R. Lajko, H. Hintner, M. Breitenbach, and A. Bito.** 2009. *UME6* is a crucial downstream target of other transcriptional regulators of true hyphal development in *Candida albicans*. *FEMS Yeast Res.* **9:**126–142.
145. **Zheng, X., and Y. Wang.** 2004. Hgc1, a novel hypha-specific G1 cyclin-related protein regulates *Candida albicans* hyphal morphogenesis. *EMBO J.* **23:**1845–1856.
146. **Zheng, X. D., R. T. Lee, Y. M. Wang, Q. S. Lin, and Y. Wang.** 2007. Phosphorylation of Rga2, a Cdc42 GAP, by CDK/Hgc1 is crucial for *Candida albicans* hyphal growth. *EMBO J.* **26:**3760–3769.
147. **Zheng, X. D., Y. M. Wang, and Y. Wang.** 2003. Ca*SPA2* is important for polarity establishment and maintenance in *Candida albicans*. *Mol. Microbiol.* **49:** 1391–1405.
148. **Zhu, W., and S. G. Filler.** 2010. Interactions of *Candida albicans* with epithelial cells. *Cell. Microbiol.* **12:** 273–282.
149. **Ziman, M., D. Preuss, J. Mulholland, J. M. O'Brien, D. Botstein, and D. I. Johnson.** 1993. Subcellular localization of Cdc42p, a *Saccharomyces cerevisiae* GTP-binding protein involved in the control of cell polarity. *Mol. Biol. Cell* **4:**1307–1316.
150. **Zou, H., H. M. Fang, Y. Zhu, and Y. Wang.** 2009. *Candida albicans* Cyr1, Cap1 and G-actin form a sensor/effector apparatus for activating cAMP synthesis in hyphal growth. *Mol. Microbiol.* **75:**579–591.

HOST-PATHOGEN INTERACTIONS (THE HOST)

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy
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10.1128/9781555817176.ch9

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Immunology of Invasive Candidiasis

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Invasive candidiasis is a life-threatening opportunistic infection and has emerged as a major cause of morbidity and mortality in critically ill patients. *Candida* spp. are the fourth most common cause of bloodstream infections in the United States, but they are a much less common cause of bloodstream infections in Europe (43). Generally, the incidence of candidemia in the intensive care unit (ICU) is 5- to 10-fold higher than in the entire hospital and more than 100-fold greater than in the general population. While the crude mortality rate of patients with candidemia is between 35 and 60%, ICU patients with candidemia have a higher mortality rate than non-ICU patients. Until recently, *Candida albicans* was by far the predominant species, causing up to two-thirds of all cases of invasive candidiasis. However, a shift toward non-*C. albicans Candida* spp., such as *C. glabrata* and *C. krusei*, with reduced susceptibility to commonly used antifungal agents was recently observed. Candidemia is also the most frequent clinical manifestation of *Candida* spp. in prosthetic-related infections (81), pediatric (8) or critically ill surgical (73) patients, and patients with different types of solid-organ transplants (23). Unfortunately, risk factors and clinical manifestations of candidiasis are not specific, and conventional culture methods such as blood culture systems lack sensitivity. Despite major advances in the field of antifungal therapy, invasive candidiasis remains a persistent public health problem (55).

This chapter highlights how the past several years have seen remarkable advances in understanding the basic cellular and immunological mechanisms underlying resistance to the fungus but also organ dysfunction and failure of recovery relating to invasive candidiasis. Current understanding of the pathophysiology underlying *Candida* infections highlights the multiple cell populations and cell-signaling pathways involved in these complex conditions, including the novel findings on the molecular connection between the failure to resolve inflammation, lack of antifungal immune resistance, and susceptibility to *Candida* infections and diseases. Chapter 10 also describes immunity to candidiasis.

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IMMUNOLOGY OF *CANDIDA*

The complex relationships of *Candida* with the vertebrate immune system are partly due to some prominent features. Among these, besides genomic microvariation (53) to adapt to environmental abiotic stress conditions (11, 26), the ability to reversibly switch from one growth form to the other in infection may have resulted in an expanded repertoire of cross-regulatory and overlapping antifungal host responses at different body sites. However, because *Candida* diseases are rare compared to the frequency of colonization, a stable host-parasite interaction is a likely condition for this successful commensal. This condition requires that the elicited immune response be strong enough to allow host survival with or without fungus elimination and to establish commensalism/persistency without excessive proinflammatory pathology. Therefore, the balance of proinflammatory and anti-inflammatory signaling is a prerequisite for successful host-fungal interactions and requires the coordinate actions of both innate and adaptive immune systems (61, 62). These new findings fit nicely within the conceptual framework of a two-component antifungal response that includes resistance, i.e., the ability to limit fungal burden, and tolerance, i.e., the ability to limit the host damage caused by either the immune response or other mechanisms. Evolutionarily conserved from plants to vertebrates (72), this new concept may help to define the best fitness in response to the fungus and its integration into new medical practices.

RESISTANCE AND TOLERANCE TO *CANDIDA*

Resistance and tolerance are two types of host defense mechanisms that increase fitness in response to fungi (87). In experimental candidiasis, both defense mechanisms are activated through the delicate equilibrium between Th/Th17 cells (which provide antifungal resistance mechanisms) and regulatory T cells (Tregs) limiting the consequences of the associated inflammatory pathology (Fig. 1 and further discussed below). Indeed, while some degree of inflammation is required for protection, particularly at mucosal tissues, during the transitional response occurring between the rapid innate and slower adaptive responses, progressive inflammation worsens disease and ultimately prevents pathogen eradication. The inflammatory response is initially mediated by cells of the innate immune system,

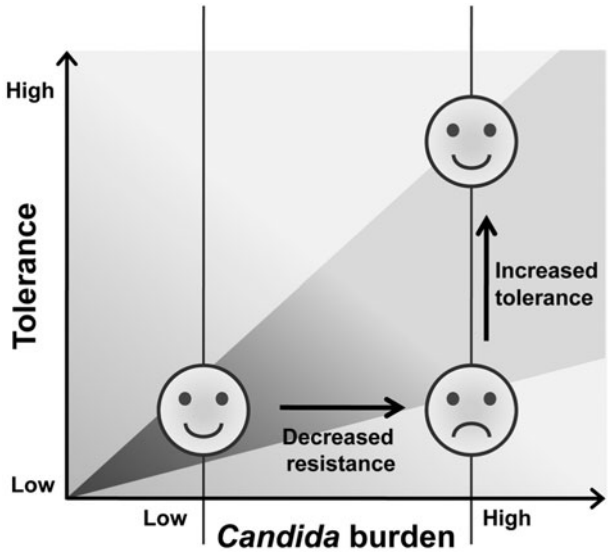

FIGURE 1 Resistance and tolerance to *Candida albicans*. The figure illustrates how the interplay between resistance, i.e., the ability to limit pathogen burden, and tolerance, i.e., the ability of the host to defend itself by limiting the damage caused by the infection, determines the ability of the host to resist fungal exposure and infection. An increased understanding of the two mechanisms in infections could aid the diagnosis and treatment of candidiasis (see text for further insights).
10.1128/9781555817176.ch9f1

followed by a later adaptive immune response, which is triggered by the signals originated by the innate immune system. The decision of how to respond will still be primarily determined by interactions between pathogens and cells of the innate immune system, but the actions of T cells will feed back into this dynamic equilibrium to suppress overzealous innate responses. Although Th1 responses driven by the interleukin 12/gamma interferon (IL-12 /IFN-γ) axis are central to protection against *Candida*, it is also an undisputed fact that patients with inborn deficits in the IL-12/IL-23/IFN-γ loop do not demonstrate increased susceptibility to *Candida* infection (17). This finding implies that other cytokine pathways may also play a role. The new entry, the Th17 pathway, which plays an inflammatory role previously attributed to uncontrolled Th1 cell reactivity and Tregs, capable of fine-tuning protective antimicrobial immunity in order to minimize harmful immune pathology, has become an integral component of the immune response to fungi. The enzyme indoleamine 2,3-dioxygenase (IDO) and kynurenines pivotally contribute to this delicate balance by providing the host with immune mechanisms adequate for protection without necessarily eliminating fungal pathogens or causing an unacceptable level of tissue damage (further discussed below). In their capacity to induce Tregs and inhibit Th17, IDO and kynurenines pivotally contribute to cell lineage decision in experimental fungal infections and revealed an unexpected potential in the control of inflammation, allergy, and Th17-driven inflammation in these infections. In this context, the Th17 pathway, which downregulates tryptophan catabolism, may instead favor pathology and serves to accommodate the seemingly paradoxical association of chronic inflammation with fungal disease (61).

CANDIDIASIS AS AN IRS-LIKE DISEASE

The inflammatory response, initiated by cells of the innate immune system, is followed by adaptive immunity, which responds to, and at the same time regulates, signals emanating from the innate system. Unresolved infection and inflammation are major epigenetic and environmental factors that contribute to chronic diseases, autoimmunity, and, in specific settings, an increased risk of cancer (20). Fungi can exploit or subvert a host's inflammatory response (48) and thus affect carriage and pathogenicity (24). The inflammatory response may serve to limit infection, but an overzealous or heightened inflammatory response may contribute significantly to the histological patterns and pathogenicity, as documented by the occurrence of severe fungal infections in patients with immune reconstitution syndrome (IRS), an entity characterized by local and systemic reactions that have both beneficial and deleterious effects on infection (74). These patients may experience intractable fungal infections despite recovery from neutropenia and the occurrence of pathogen-specific immunity. Intriguingly, IRS responses are also found in immunocompetent individuals and after rapid resolution of immunosuppression, indicating that inflammatory responses can result in quiescent or latent infections manifesting as opportunistic mycoses. Thus, although host immunity is crucial in eradicating infection, immunological recovery can also be detrimental and may contribute to worsening disease in opportunistic and non-opportunistic infections. This suggests that fungal outgrowth does not occur as a result of a weak or inefficient immune response. For *Candida*, the failure to resolve inflammation associated with defective fungal clearance characterizes both chronic mucocutaneous candidiasis (CMC) and chronic disseminated candidiasis (CDC). CMC is a primary immunodeficiency presenting as an inability to clear yeasts, mostly *C. albicans*, that consequently persist and recur in infections of the skin, nails, and mucous membranes (36). Most CMC patients also develop accompanying endocrine and inflammatory disorders that suggest an underlying deregulation of the inflammatory and immune responses. CDC is typically observed during neutrophil recovery in patients with acute leukemia and requires protracted antifungal therapy. However, the efficacy of adjuvant corticosteroid therapy in these patients supports the pathophysiological hypothesis that CDC belongs to the spectrum of fungus-related immune reconstitution inflammatory syndrome (34). All these observations highlight a truly bipolar nature of the inflammatory process in infection. Early inflammation prevents or limits infection, but an uncontrolled response may eventually oppose disease eradication. A main implication of these findings is that, at least in specific clinical settings, it is an exaggerated inflammatory response that likely compromises a patient's ability to eradicate infection, and not an "intrinsic" susceptibility to infection that determines a state of chronic or intractable disease.

SHAPING IMMUNITY TO CANDIDA: FROM FUNGAL RECOGNITION TO IMMUNE ACTIVATION

Innate immunity and acquired cell-mediated immunity have been acknowledged as the primary mediators of host resistance to *C. albicans* (2, 61, 62). With the recognition of the reciprocal influences between the innate and the adaptive Th immunity, it is now firmly established that an

integrated immune response determines the lifelong commensalism of the fungus at the mucosal level, as well as the transition from mucosal saprophyte to pathogen. Receptors on phagocytes not only mediate downstream intracellular events related to clearance but also participate in complex and disparate functions related to immunomodulation and activation of immunity, depending on cell type. Therefore, in order to achieve optimal activation of antigen-specific adaptive immunity, it is first necessary to activate the pathogen detection mechanisms of the innate immune response.

Innate Immune Receptors

Most of the innate mechanisms are inducible upon infection, and their activation requires specific recognition of invariant evolutionarily conserved molecular structures shared by large groups of pathogens by a set of pattern recognition receptors (PRRs) that directly recognize fungal molecules, including Toll-like receptors (TLRs), C-type lectin receptors (CRLs), the nucleotide binding domain leucine-reach repeat-containing receptors (NLRs), and the galectin family (49, 67) (Fig. 2). In response to *Candida*, PRRs on the surface of professional phagocytes recognize different fungal components, such as mannans, mannoproteins, β-glucans, and chitin. Ligand recognition by TLRs is followed by activation of myeloid differentiation factor 88 (MyD88)- or Toll–IL-1 receptor domain-containing adapter-inducing beta interferon (TRIF)-dependent pathways leading to the stimulation of proinflammatory cytokines and type I interferons, respectively. Several TLRs, including TLR2, TLR4, and TLR9, mediate recognition of *Candida* pathogen-associated molecular patterns (PAMPs) (5, 29, 49). TLR2 from myeloid cells recognizes the phospho-lipomannan component of the *Candida* cell wall (28). TLR2-deficient animals have either an increased (82) or decreased (3, 52) susceptibility to systemic candidiasis. The immunosuppresive effect induced by TLR2, which explains the increased resistance of TLR2-deficient mice to disseminated candidiasis, was found to be mediated through the generation of Tregs with immunosuppressive potential (52). TLR4 recognizes short linear O-bound mannans of *C. albicans* and induces proinflammatory cytokines production (50). However, the role of TLR4 in the host defense against invasive candidiasis is still debated, due to variable results from different laboratories. Of interest, a recent study has helped to explain the apparent contradictory results obtained with TLR4. It was found that the sequential activation of the MyD88 and the TRIF pathways downstream TLR4 finely modulated the migratory and functional activities of Tregs at the infection sites, thus accounting for the proper control of fungal growth and Th immune activation (12). This study suggests that the generation of protective immunity to *C. albicans* relies on the sequential activation of the MyD88 and TRIF pathways with distinct, nonoverlapping roles. As a matter of fact, type I interferons have recently been reported to affect susceptibility to invasive candidiasis (4a). TLR9 can recognize fungal DNA (47). However, the exact contribution of fungal recognition by TLR9 to overall *Candida* immunity is unclear.

The CLRs are a family of PRRs that recognize polysaccharide structures, and they include several receptors implicated in fungal recognition, such as mannose receptor (MR; CD206), dectin-1, dectin-2, DC-SIGN (CD209), Mincle, and the circulating MBL (5, 6). These receptors have been shown to be central for fungal recognition and for induction of the innate antifungal immune response. The MR recognizes branched N-bound mannans from *C. albicans* (50) and mediates intracellular signals leading to cytokine production and the induction of Th17 responses (78). However, MR does not seem to affect susceptibility to infection in vivo (33). Dectin-1 carries a cytoplasmic immunoreceptor tyrosine-based activation-like domain, whereas dectin-2 and Mincle selectively associate with the Fcγ receptor common chain to elicit a cytokine response (5, 18, 71). Dectin-1, which is predominantly expressed in myeloid cells, is considered the main receptor stimulated by β-1,3-linked glucan to execute fungal phagocytosis in innate cells (57). Dectin-1 signaling proceeds through the Src/Syk kinase/caspase recruitment domain-containing protein 9 (CARD9) pathway to activate both the canonical and noncanonical NF-κB pathways (19). The nature and magnitude of downstream output responses resulting from dectin-1 activation depend on the cell type and the respective microenvironment. Infection with *C. albicans*

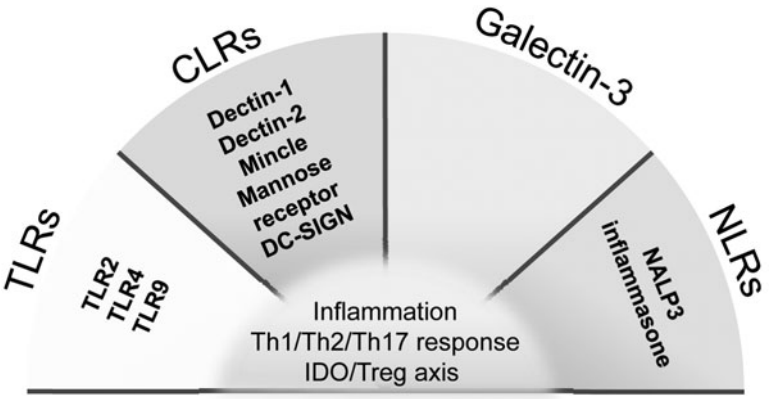

FIGURE 2 Major PRRs involved in recognition of *Candida albicans* and activation of subsequent inflammatory/Th responses. The exploitation of the IDO/Treg axis for regulatory mechanisms may have allowed the commensal to coevolve with the mammalian immune system and to survive in conditions of high-threat inflammation (see text for a detailed description). 10.1128/9781555817176.ch9f2

induces protective dectin-1/CARD9-dependent antifungal responses (35). Dectin-1- and similarly CARD9-deficient mice are more susceptible to disseminated candidiasis (22, 77). These data suggest a role for dectin-1 in antifungal immunity, either directly or through collaborative signaling with other PRRs, such as TLR2 (57). The recent finding that dectin-1 signaling and cytokine induction are inhibited by the tetraspanin CD37 (80) demonstrates the importance of fine-tuning the inflammatory signals from dectin-1.

Dectin-2 recognizes high-mannose structures of many fungi, with a high affinity for hyphal forms (71). Dectin-2 selectively associates with the FcγR common chain to induce cytokine release through Syk and CARD9 activation (59). In a mouse model of *Candida* infection, the blockade of dectin-2 fails to impair survival, whereas it abrogates the *Candida*-induced Th17 response (70). In conjunction with a lack of dectin-1, functional inactivation of dectin-2 diminishes the protective Th1 response to *Candida* spp. (59). Mincle is a receptor that senses nonhomeostatic cell death and thereby induces the production of inflammatory cytokines to drive the infiltration of neutrophils into damaged tissue (84). Upon *C. albicans* recognition, Mincle activates macrophages to mount a strong inflammatory response, although it is not required for *Candida* phagocytosis (83). A lack of Mincle increases fungal burdens in a mouse model of *Candida* infection (83). In contrast, another study suggests that Mincle contributes only to *Malassezia* sp. recognition among 50 other fungal species tested (85). DC-SIGN and its related receptor DC-SIGNR are primarily expressed on mature dendritic cells (DCs) and recognize high-mannose structures in a calcium-dependent way (7). DC-SIGN mediates uptake of fungal particles by DCs and recognizes mannans from *C. albicans* (7). Galectin-3 is a receptor mainly expressed by macrophages, and it has been shown to be involved in the recognition of the beta-mannosides of *C. albicans*, in close collaboration with TLR2 (31).

In addition to the mainly cell membrane-bound TLRs and CLRs, mammalian host defense has built a second line of recognition receptors located intracytoplasmatically, which, for fungi, is represented by the receptors of the NLR family (5, 49). NLRs sense nonmicrobial danger signals—that is, xenocompounds or molecules that when recognized alert the immune system to hazardous environments, perhaps independently of a microbial trigger—and form large cytoplasmic complexes called inflammasomes that link the sensing of fungal products and metabolic stress to the proteolytic activation of the proinflammatory cytokines IL-1β and IL-18. The NLR family pyrin domain-containing 3 (NALP3) inflammasome has been associated with several autoinflammatory conditions and can direct adaptive immune responses (9). The inflammasomes are protein complexes that upon recognition of a microbial PAMP or an endogenous danger signal (e.g., ATP or uric acid) induce activation of the cysteine protease caspase-1, which, in turn, processes pro-IL-1β and pro-IL-18 into the bioactive cytokines (41, 42). An important role for the NLRP3 inflammasome for anti-*Candida* host defense has been proposed, as NLRP3-deficient mice are more susceptible to systemic *Candida* infection (27).

Dendritic Cells

DCs are uniquely adept at decoding the fungus-associated information and translating it into qualitatively different adaptive T-cell immune responses (63). The finding that methamphetamine, whose chronic abuse has reached epidemic proportions, potently inhibits *Candida* processing and killing by DCs (76) points to the unique and indispensable role of DCs in antifungal immunity. Indeed, impaired DC maturation and function have been associated with disease in patients with CMC (68). The exploitation of distinct recognition receptors in DCs determines the full range of the host's immune relationships with *C. albicans* (63, 64). Similarly to what has been reported with mononuclear phagocytes (49), distinct intracellular signaling pathways are likely to play a role in the variegation of antifungal immune responses by DCs. The ability of a given DC subset to respond with flexible activating programs to the different stimuli, as well as the ability of different subsets to convert into each other, confers unexpected plasticity on the DC system (63, 64). As a matter of fact, the capacity of DCs to initiate different adaptive anticandidal immune responses depended upon specialization and cooperation between DC subsets as well as the activation of distinct intracellular signaling pathways (4).

A wealth of evidence indicates that DC immunogenicity/tolerogenicity is not a characteristic of a specific subset or lineage of DCs but an environmentally acquired feature. In this regard, the tryptophan metabolic pathway (see below) pivotally contributed to DC regulation, such that tolerance and Treg induction could be mediated by plasmacytoid DCs (pDCs) expressing IDO (54). In response to fungi, IDO expression conferred tolerogenic properties to DCs (88) such that *Candida*-pulsed, IDO-expressing gut DCs ameliorated experimental colitis (4). Thus, multiple, functionally distinct receptor/signaling pathways in DCs ultimately affecting the local Th/Treg balance could be successfully exploited for either commensalism or pathogenicity, a finding suggesting a high degree of coevolution of the mammalian host and *C. albicans* and the resultant symbiosis between the two.

Acquired Antibody- and Cell-Mediated Immunity

The success of passive antibody in preventing and treating fungal infections in experimental models and the success of certain vaccines that elicit protective antibody strongly indicate that some antibody responses can make a decisive contribution to host defense against medically important fungi (56). Protective antibodies have been described for *C. albicans*. In recent years two antibodies have entered clinical evaluation for fungal diseases. In addition to classical mechanisms of antibody action, additional mechanisms have been revealed, including inhibition of growth and germination, biofilm formation, direct antifungal effects, and alteration of intracellular trafficking of fungi.

A consensus has emerged that the inability of immune sera to mediate protection against fungi reflects inadequate amounts of protective antibody and/or the simultaneous presence of protective and nonprotective antibodies. Nonetheless, much remains to be learned about the nature of protective antibodies and the relationship between the natural antibody response and resistance and susceptibility to fungal pathogens, since antibody responses can be a marker of disease rather than immunity. Additionally, there is currently insufficient evidence to indicate how antibodies mediate their protective effects at the different body sites in infections.

Serological and skin reactivity surveys indicate the development of acquired cell-mediated immunity to fungi. Lymphocytes from healthy subjects show strong proliferative responses after stimulation with *Candida* antigens and produce a number of different cytokines (61). Underlying acquired immunity to *C. albicans*, such as the expression of

positive delayed-type hypersensitivity (DTH), is demonstrable in adult immunocompetent individuals.

Th1/Th2 Cells

Generation of a dominant Th1 response driven by IL-12 is essentially required for the expression of protective immunity to *Candida*. Through the production of the signature cytokine IFN-γ and help for opsonizing antibodies, the activation of Th1 cells is instrumental in the optimal activation of phagocytes at sites of infection. Therefore, the failure to deliver activating signals to effector phagocytes may predispose patients to overwhelming infections, limit the therapeutic efficacy of antifungals and antibodies, and favor persistency and/or commensalism (62). IL-4 acts as the most potent proximal signal for commitment to Th2 reactivity that dampens protective Th1 responses and favors fungal allergy. IL-4 may both deactivate and activate phagocytes and DCs for certain specialized function; for instance, it may inhibit the antifungal effector activities of phagocytes, yet may promote IL-12 production by DCs (62). Thus, the most important mechanism underlying the inhibitory activity of IL-4 in infections relies on its ability to act as the most potent proximal signal for commitment to Th2 reactivity that dampens protective Th1 responses. However, susceptibility to candidiasis may not always be associated with an overt production of IL-4 (36).

Over the past several years, the demise of a Th1/Th2 dichotomy paradigm has been accompanied by a renaissance in probing the basic tenets of $CD4^+$ T-cell biology. As a result, instead of only two distinct "fates" for developing T cells, research has identified alternative fates and more flexibility in T-cell cytokine production than previously envisioned (90). Th17 cells are now thought to be a separate lineage of effector Th cells contributing to immune pathogenesis previously attributed to the Th1 lineage (30).

Th17 Cells

Accumulating data support a role for Th17 cells and Th17 cytokines in inflammatory processes and in animal models of autoimmunity or inflammation (32, 38, 46, 90). Th17 cells have an important function in the host defense response against extracellular pathogens, but they also have become notorious for their role in the pathogenesis of many autoimmune and allergic disorders. Th17 cells are a separate lineage of effector Th cells contributing to immune pathogenesis previously attributed to the Th1 lineage (30). Interestingly, though, Th17 cells are found early during the initiation of an immune response and have been reported to be involved in a broad range of both Th1- and Th2-dominated immune responses. Several lines of evidence further support the notion of a reciprocal relationship between the transcription factor forkhead box P3 (FoxP3)-positive Tregs and Th17 cells. Emerging data on the mechanism by which Th17 cells induce tissue inflammation suggest that Th17 cells first infiltrate the site of tissue inflammation and then recruit other proinflammatory effector T cells (including Th1 cells) and innate cells (including neutrophils) to sites of tissue inflammation. As IL-17 receptors are widely expressed on parenchymal/tissue cells and IL-17 induces production of IL-1, IL-6, tumor necrosis factor, matrix metalloproteinases, IL-8, and chemokines, these mediators coordinate infiltration of other cell types to the site of inflammation and mediate massive tissue inflammation at the site where IL-17 is abundantly produced.

Th17 cells are induced in candidiasis, through TLR- and non-TLR-dependent signaling (12, 35, 58, 70, 78, 86). Th17 cells are present in the human T-cell memory repertoire to the fungus (1, 15), and defective Th17 cell differentiation has been linked to CMC in patients with primary immunodeficiencies (45). Although recent evidence supports the importance of the dectin-1/IL-17 axis in human mucocutaneous candidiasis (16), both positive and negative effects on immune resistance have been attributed to Th17 and IL-17 receptor signaling in experimental disseminated candidiasis (10, 25, 70, 86). Thus, the role of IL-17 and Th17 cells in immunity versus pathology in fungal infections and diseases remains controversial (87). It is likely that the protective versus disease-promoting effect of the IL-17/Th17 pathway may depend on the stage and site of infection. Early IL-17 is able to exert some forms of antifungal resistance, via IL-22 (see below), defensins, and neutrophils, while the failure to downregulate microbe-induced expression of IL-17 could eventually be one major link connecting infection with chronic inflammation.

The mechanisms that linked inflammation to chronic infection have been credited to the offending potential of IL-17A that, although promoting neutrophil recruitment, impeded the timely restriction of neutrophil inflammatory potential, thus preventing optimal protection from occurring (66). IL-17A also activated the inflammatory program of neutrophils by counteracting the IFN-γ-dependent activation of IDO, known to limit the inflammatory status of neutrophils, as well as by inducing the release of metalloproteinases and oxidants, which likely accounts for the high inflammatory pathology and tissue destruction associated with Th17 cell activation. These new findings provide a molecular connection between the failure to resolve inflammation and lack of antifungal immune resistance and point to strategies for immune therapy of fungal infections that attempt to limit inflammation to stimulate an effective immune response. More generally, the Th17 pathway could be involved in the immunopathogenesis of chronic fungal diseases where persistent fungal antigens may maintain immunological dysreactivity.

Despite the excitement raised by the new findings, much remains to be learned, including the dependency of Th17 cells on the plasticity of human $CD4^+$ T-cell differentiation and the relative contributions of the various populations of IL-17-producing cells to the pathogenesis of infections and diseases caused by fungi. In this regard, Th17 cells also produce IL-22, a member of the IL-10 family of cytokines which has been shown to play a more important role than IL-17 in host defense in the lung and gut (89). Recent findings suggest that the IL-23/IL-22/defensin pathway is crucially involved in the control of fungal growth at mucosal and nonmucosal sites, particularly in conditions of Th1 deficiency (13) (Fig. 3). The IL-23/IL-22 axis controls the initial fungal growth and tissue homeostasis, likely exploiting primitive antifungal effector defense mechanisms, while adaptive Th1/Treg cells prevent fungal dissemination and provide memory and tolerance (13). With a functional Th1 pathway, the IL-23/IL-22 axis is even dispensable. However, in a defective Th1 pathway, a heightened innate IL-22 response provides resistance. These findings suggest that functionally distinct "modules" of immunity evolved to provide resistance, i.e., the ability to limit fungal burden, or tolerance, i.e., the ability to limit host damage in response to *Candida* (87). Of interest, the IL-22 pathway, more than the Th1/Treg pathway, is exploited by nonpathogenic yeasts, such as *C. krusei* and *Saccharomyces cerevisiae* (13). Considering the burden of nosocomial infections by opportunistically pathogenic yeast species, the new finding may offer new

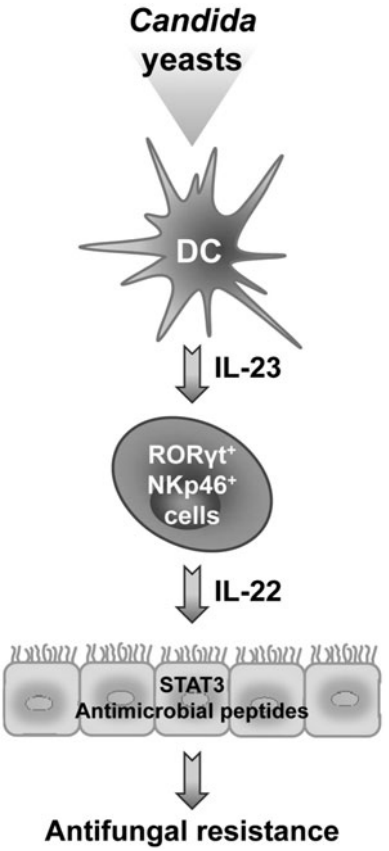

FIGURE 3 IL-22 defines a novel immune pathway of antifungal resistance. DC-derived IL-23 promotes the production of IL-22 from cryptopatch CD3-NKp46$^+$ expressing RORγt, IL-23R, aryl hydrocarbon receptor, CCR5, and CCR7, indicating their similarity to gut RORγt$^+$ NKp46$^+$ cells and cryptopatch lymphoid tissue inducer-like cells that are expanded by commensals. IL-22 targets epithelial cells for STAT3 activation and, together with IL-17A, for antimicrobial peptide production (13). 10.1128/9781555817176.ch9f3

interpretative clues to explain why some individuals are at high risk for yeast infections. That memory IL-22$^+$ CD4$^+$ cells specific for *C. albicans* are present in humans (39) and are defective in patients with CMC (14) are findings consistent with the new data. Interestingly, the fact that IL-22 production in the gut is driven by commensals also provides novel mechanistic insights on how antibiotic therapy and iatrogenic immunosuppression are major predisposing factors in candidiasis and, more generally, how bacterial-fungal population dynamics affect gut homeostasis and inflammatory diseases. These new findings further help to explain the susceptibility to bacterial and fungal infections in patients with autosomal dominant hyper-immunoglobulin E syndrome (AD-HIES) (40, 45). Due to dominant-negative mutations of STAT3, AD-HIES patients have a defective Th17 response that is likely amplified on epithelia, where STAT3 mutations compromise the IL-22 effects. Thus, the defective Th1 response in AD-HIES patients (51) and the low IL-22 production in CMC (14) are clinical features consistent with the Th1-supporting role of and IL-22 production by Th17 cells (37, 60).

Treg Cells

A number of clinical observations suggest an inverse relationship between IFN-γ and IL-10 production in patients with fungal infections. High levels of IL-10, negatively affecting IFN-γ production, are detected in chronic candidal diseases (14). However, solid evidence demonstrating a causal role for IL-10 in susceptibility to fungal infections is lacking. It has been suggested that, rather than causing the infection, IL-10 production may be a consequence of the infection (65). This would predict that in the case of chronic fungal infections, characterized by a state of chronic inflammation, IL-10 could be the homeostatic host-driven response aimed at keeping, however possible, inflammation under control. In murine candidiasis, CD4$^+$ CD25$^+$ Treg cells prevent excessive inflammation but enable fungal persistence in the gastrointestinal tract, which underlies the onset of durable antifungal protection. However, as the Treg response may handicap the efficacy of protective immunity, the consequence of Treg activity is less damage to the host but also fungal persistence. Because both the recovery of *C. albicans* from the gastrointestinal tract and the detection of underlying Th1 reactivity, such as DTH and lymphoproliferation, can fluctuate in healthy subjects, it is likely that Tregs mediate tolerance to the fungus at the site of colonization. This may have allowed fungal persistence and the occurrence of memory immunity to a commensal. CMC, although encompassing a variety of clinical entities (36), has been associated with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy, a condition in which Treg induction is defective (69). The different Treg cell populations may have the capacity to influence the emergence or function of one another. This was best illustrated in murine candidiasis, in which fungal growth, inflammatory immunity, and tolerance to the fungus were all controlled by the coordinate activation of naturally occurring Treg (nTreg) cells—limiting early inflammation at the sites of infection—and pathogen-induced Treg (iTreg) cells, which regulated the expression of adaptive Th immunity in secondary lymphoid organs. nTreg cells required the TRIF pathway for migration to inflamed sites, where the MyD88 pathway then restrained their suppressive function. Subsequent inflammatory Th1-type immunity was modulated by iTreg cells, which required the TRIF pathway as well, and which acted through activation of IDO in DCs and Th17 cell antagonism (12).

Collectively, these observations suggest that the capacity of Tregs to inhibit aspects of innate and adaptive immunity is pivotal in their regulatory function and further support the concept of "protective tolerance" to fungi, implying that a host's immune defense may be adequate for protection without necessarily eliminating fungal pathogens—which would impair immune memory—or causing an unacceptable level of tissue damage (66).

IDO AS A MAMMALIAN REGULATOR OF RESISTANCE AND TOLERANCE TO *CANDIDA*

The enzyme IDO has a complex role in immunoregulation in infection, pregnancy, autoimmunity, transplantation, and neoplasia (21). IDO catalyzes the first and limiting step in the kynurenine pathway of tryptophan catabolism. Initially recognized in infection because of antimicrobial activity ("tryptophan starvation" of intracellular parasites), IDO is more importantly and widely involved in immune homeostasis of the mammalian host, and may even represent an

evasion mechanism for microbes that establish commensalism or chronic infection (88). Within a conceptual framework from different areas of immune regulation, the many and apparently disparate functions of IDO in infection are now being reconciled, emphasizing a quite complex role for a metabolic pathway that appears to be conserved—though evolved in function—through the last 600 million years of evolution.

IDO and the downstream enzymes in the metabolic pathway of tryptophan degradation have a central role in chronic infection (88). Present in innate immune cells such as macrophages, neutrophils, and epithelial cells, IDO is a key player in the suppression of acute inflammatory responses. Furthermore, the activation of the amino acid starvation response was recently reported to inhibit Th17 differentiation in vivo (75), and its expression by pDCs enables the onset of tolerance in adaptive immunity. Thus, in addition to direct effector activities, largely involving tryptophan deprivation, IDO and the other enzymes of the metabolic pathway contribute immunoactive molecules to the generation of regulatory Tregs with anti-inflammatory and tolerogenic activities. These findings establish a mutual interaction between DCs and Tregs for the upkeep of immunological tolerance and would predict that in certain infectious settings, any direct IDO antimicrobial activity resulting from tryptophan starvation could be somewhat blunted by the induction of tolerogenic responses that would allow pathogens to take advantage of the "immune privilege" normally reserved to mammalian hosts (44).

In experimental candidiasis, IDO and kynurenines pivotally contribute to the delicate inflammatory/anti-inflammatory balance by providing the host with immune mechanisms adequate for protection without necessarily eliminating fungal pathogens or causing an unacceptable level of tissue damage (65, 87). More recently, while capable of inducing the Foxp3-encoding gene transcriptionally, tryptophan catabolites were also found to suppress the gene encoding RORγt (retinoid-related orphan receptor γt), the Th17 lineage specification factor (12). Thus, in their capacity to induce Tregs and inhibit Th17, IDO and kynurenines pivotally contribute to cell lineage decision in experimental candidiasis and revealed an unexpected potential in the control of inflammation and Th17-driven inflammation. In this context, the Th17 pathway, which downregulates tryptophan catabolism, may instead favor pathology and serves to accommodate the seemingly paradoxical association of chronic inflammation with fungal diseases (61, 66).

The implications for IDO in immunoregulation are manifold. As *C. albicans* is a commensal of the human gastrointestinal and genitourinary tracts and IFN-γ is an important mediator of protective immunity to the fungus, the IFN-γ/IDO axis may accommodate fungal persistence in a host environment rich in IFN-γ. Because IFN-γ is a potent IDO activator, this suggests the existence of an IFN-γ/IDO-dependent pathway leading to sequential Th1/Treg cell activation in infection. In its ability to induce Th1 immunity within a regulatory environment and to prevent Th17 development, IDO expression may correlate with the occurrence of local tolerogenic responses. Alternatively, the high levels of IL-10 production may be a consequence of IDO activation by the fungus, impairing antifungal Th1 immunity and thus favoring persistent infection. Intriguingly, the fact that hyphae, more than yeasts, activate the expression of IDO further suggests that differential sensing of fungal morphotypes through distinct recognition receptors may promote distinct immune responses and that fungal hyphae, more than yeasts, may promote tolerance and thus contribute to commensalism and eventually to immunoevasion.

CONCLUSIONS AND FUTURE PERSPECTIVES

The new discoveries in the field of *Candida* immunology have offered new grounds for a better comprehension of cells and immune pathways that are amenable to manipulation in patients with or at risk of infections (79). The intricate cross talk provided by temporal changes in mediators, metabolites, and cell phenotypes underlines the coordinated processes beyond the dysregulated chaos in which fungal infection and disease are perceived. Applying systems biology approaches to these complex processes has permitted better appreciation of the effectiveness or harm of treatments, and also has allowed development not only of better-directed but also of more appropriately timed strategies to improve outcomes from this life-threatening infection. Our increasing understanding of the basic mechanisms that dictate development and function of Th17 cells, as well as our better knowledge of how Th17/Tregs regulate each other as well as other immune and nonimmune cells, provides guidelines for rational design of novel immunomodulatory therapies that limit inflammation in order to stimulate an effective immune response. Tryptophan metabolites and Th17 inhibitors are likely candidates as potent regulators capable of taming overzealous or heightened inflammatory host responses to the benefit of pathogen control and host survival. Notwithstanding the redundancy and overlapping repertoire of antifungal effector mechanisms, the pivotal role of different types of Tregs in the control of Th1/Th2 inflammatory responses as well as in Th17 antagonism suggests that manipulation of Tregs could be a promising therapeutic approach devoid of risks associated with interference with homeostatic mechanisms of the immune system.

These studies were supported by the Specific Targeted Research Project "ALLFUN" (FP7–HEALTH–2009-260338) and the Italian Project AIDS 2010 by ISS (Istituto Superiore di Sanità - Contract number 40H40).

REFERENCES

1. **Acosta-Rodriguez, E. V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani.** 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* **8:**639–646.
2. **Ashman, R. B.** 2008. Protective and pathologic immune responses against *Candida albicans* infection. *Frontiers Biosci.* **13:**3334–3351.
3. **Bellocchio, S., C. Montagnoli, S. Bozza, R. Gaziano, G. Rossi, S. S. Mambula, A. Vecchi, A. Mantovani, S. M. Levitz, and L. Romani.** 2004. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J. Immunol.* **172:** 3059–3069.
4. **Bonifazi, P., T. Zelante, C. D'Angelo, A. De Luca, S. Moretti, S. Bozza, K. Perruccio, R. G. Iannitti, G. Giovannini, C. Volpi, F. Fallarino, P. Puccetti, and L. Romani.** 2009. Balancing inflammation and tolerance in vivo through dendritic cells by the commensal *Candida albicans*. *Mucosal Immunol.* **2:**362–374.

4a. **Bourgeois, C., O. Majer, I. E. Frohner, I. Lesiak-Markowicz, K. S. Hildering, W. Glaser, S. Stockinger, T. Decker, S. Akira, M. Müller, and K. Kuchler.** 2011. Conventional dendritic cells mount a type I IFN response

against *Candida* spp. requiring novel phagosomal TLR7-mediated IFN-β signaling. *J. Immunol.* **186:**3104–3112.
5. **Bourgeois, C., O. Majer, I. E. Frohner, L. Tierney, and K. Kuchler.** 2010. Fungal attacks on mammalian hosts: pathogen elimination requires sensing and tasting. *Curr. Opin. Microbiol.* **13:**401–408.
6. **Brown, G. D.** 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* **6:**33–43.
7. **Cambi, A., M. G. Netea, H. M. Mora-Montes, N. A. Gow, S. V. Hato, D. W. Lowman, B. J. Kullberg, R. Torensma, D. L. Williams, and C. G. Figdor.** 2008. Dendritic cell interaction with *Candida albicans* critically depends on N-linked mannan. *J. Biol. Chem.* **283:** 20590–20599.
8. **Castagnola, E., and S. Buratti.** 2009. Clinical aspects of invasive candidiasis in paediatric patients. *Drugs* **69**(Suppl. 1)**:**45–50.
9. **Chen, G., M. H. Shaw, Y. G. Kim, and G. Nunez.** 2009. NOD-like receptors: role in innate immunity and inflammatory disease. *Annu. Rev. Pathol.* **4:**365–398.
10. **Conti, H. R., F. Shen, N. Nayyar, E. Stocum, J. N. Sun, M. J. Lindemann, A. W. Ho, J. H. Hai, J. J. Yu, J. W. Jung, S. G. Filler, P. Masso-Welch, M. Edgerton, and S. L. Gaffen.** 2009. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J. Exp. Med.* **206:**299–311.
11. **Cooney, N. M., and B. S. Klein.** 2008. Fungal adaptation to the mammalian host: it is a new world, after all. *Curr. Opin. Microbiol.* **11:**511–516.
12. **De Luca, A., C. Montagnoli, T. Zelante, P. Bonifazi, S. Bozza, S. Moretti, C. D'Angelo, C. Vacca, L. Boon, F. Bistoni, P. Puccetti, F. Fallarino, and L. Romani.** 2007. Functional yet balanced reactivity to *Candida albicans* requires TRIF, MyD88, and IDO-dependent inhibition of Rorc. *J. Immunol.* **179:**5999–6008.
13. **De Luca, A., T. Zelante, C. D'Angelo, S. Zagarella, F. Fallarino, A. Spreca, R. G. Iannitti, P. Bonifazi, J. C. Renauld, F. Bistoni, P. Puccetti, and L. Romani.** 2010. IL-22 defines a novel immune pathway of antifungal resistance. *Mucosal Immunol.* **3:**361–373.
14. **Eyerich, K., S. Rombold, S. Foerster, H. Behrendt, H. Hofmann, J. Ring, and C. Traidl-Hoffmann.** 2007. Altered, but not diminished specific T cell response in chronic mucocutaneous candidiasis patients. *Arch. Dermatol. Res.* **299:**475–481.
15. **Fenoglio, D., A. Poggi, S. Catellani, F. Battaglia, A. Ferrera, M. Setti, G. Murdaca, and M. R. Zocchi.** 2009. Vδ1 T lymphocytes producing IFN-γ and IL-17 are expanded in HIV-1-infected patients and respond to *Candida albicans*. *Blood* **113:**6611–6618.
16. **Ferwerda, B., G. Ferwerda, T. S. Plantinga, J. A. Willment, A. B. van Spriel, H. Venselaar, C. C. Elbers, M. D. Johnson, A. Cambi, C. Huysamen, L. Jacobs, T. Jansen, K. Verheijen, L. Masthoff, S. A. Morre, G. Vriend, D. L. Williams, J. R. Perfect, L. A. Joosten, C. Wijmenga, J. W. van der Meer, G. J. Adema, B. J. Kullberg, G. D. Brown, and M. G. Netea.** 2009. Human dectin-1 deficiency and mucocutaneous fungal infections. *N. Engl. J. Med.* **361:**1760–1767.
17. **Filipe-Santos, O., J. Bustamante, A. Chapgier, G. Vogt, L. de Beaucoudrey, J. Feinberg, E. Jouanguy, S. Boisson-Dupuis, C. Fieschi, C. Picard, and J. L. Casanova.** 2006. Inborn errors of IL-12/23- and IFN-gamma-mediated immunity: molecular, cellular, and clinical features. *Semin. Immunol.* **18:**347–361.
18. **Graham, L. M., and G. D. Brown.** 2009. The Dectin-2 family of C-type lectins in immunity and homeostasis. *Cytokine* **48:**148–155.
19. **Gringhuis, S. I., J. den Dunnen, M. Litjens, M. van der Vlist, B. Wevers, S. C. Bruijns, and T. B. Geijtenbeek.** 2009. Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-κB activation through Raf-1 and Syk. *Nat. Immunol.* **10:**203–213.
20. **Grivennikov, S. I., F. R. Greten, and M. Karin.** Immunity, inflammation, and cancer. *Cell* **140:**883–899.
21. **Grohmann, U., F. Fallarino, and P. Puccetti.** 2003. Tolerance, DCs and tryptophan: much ado about IDO. *Trends Immunol.* **24:**242–248.
22. **Gross, O., H. Poeck, M. Bscheider, C. Dostert, N. Hannesschlager, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, A. Mocsai, J. Tschopp, and J. Ruland.** 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* **459:**433–436.
23. **Grossi, P. A.** 2009. Clinical aspects of invasive candidiasis in solid organ transplant recipients. *Drugs* **69**(Suppl. 1)**:**15–20.
24. **Haas-Stapleton, E. J., Y. Lu, S. Hong, M. Arita, S. Favoreto, S. Nigam, C. N. Serhan, and N. Agabian.** 2007. *Candida albicans* modulates host defense by biosynthesizing the pro-resolving mediator resolvin E1. *PLoS One* **2:**e1316.
25. **Huang, W., L. Na, P. L. Fidel, and P. Schwarzenberger.** 2004. Requirement of interleukin-17A for systemic anti-*Candida albicans* host defense in mice. *J. Infect. Dis.* **190:** 624–631.
26. **Hube, B.** 2009. Fungal adaptation to the host environment. *Curr. Opin. Microbiol.* **12:**347–349.
27. **Joly, S., N. Ma, J. J. Sadler, D. R. Soll, S. L. Cassel, and F. S. Sutterwala.** 2009. Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. *J. Immunol.* **183:**3578–3581.
28. **Jouault, T., S. Ibata-Ombetta, O. Takeuchi, P. A. Trinel, P. Sacchetti, P. Lefebvre, S. Akira, and D. Poulain.** 2003. *Candida albicans* phospholipomannan is sensed through Toll-like receptors. *J. Infect. Dis.* **188:**165–172.
29. **Jouault, T., A. Sarazin, M. Martinez-Esparza, C. Fradin, B. Sendid, and D. Poulain.** 2009. Host responses to a versatile commensal: PAMPs and PRRs interplay leading to tolerance or infection by *Candida albicans*. *Cell. Microbiol.* **11:**1007–1015.
30. **Kaufmann, S. H., and V. K. Kuchroo.** 2009. Th17 cells. *Microbes Infect.* **11:**579–583.
31. **Kohatsu, L., D. K. Hsu, A. G. Jegalian, F. T. Liu, and L. G. Baum.** 2006. Galectin-3 induces death of *Candida* species expressing specific beta-1,2-linked mannans. *J. Immunol.* **177:**4718–4726.
32. **Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo.** 2009. IL-17 and Th17 cells. *Annu. Rev. Immunol.* **27:**485–517.
33. **Lee, S. J., N. Y. Zheng, M. Clavijo, and M. C. Nussenzweig.** 2003. Normal host defense during systemic candidiasis in mannose receptor-deficient mice. *Infect. Immun.* **71:**437–445.
34. **Legrand, F., M. Lecuit, B. Dupont, E. Bellaton, M. Huerre, P. S. Rohrlich, and O. Lortholary.** 2008. Adjuvant corticosteroid therapy for chronic disseminated candidiasis. *Clin. Infect. Dis.* **46:**696–702.
35. **Leibundgut-Landmann, S., O. Gross, M. J. Robinson, F. Osorio, E. C. Slack, S. V. Tsoni, E. Schweighoffer, V. Tybulewicz, G. D. Brown, J. Ruland, and E. S. C. Reis.** 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* **8:**630–638.
36. **Lilic, D.** 2002. New perspectives on the immunology of chronic mucocutaneous candidiasis. *Curr. Opin. Infect. Dis.* **15:**143–147.
37. **Lin, L., A. S. Ibrahim, X. Xu, J. M. Farber, V. Avanesian, B. Baquir, Y. Fu, S. W. French, J. E. Edwards, Jr., and B. Spellberg.** 2009. Th1-Th17 cells mediate

protective adaptive immunity against *Staphylococcus aureus* and *Candida albicans* infection in mice. *PLoS Pathog*. **5:** e1000703.
38. **Littman, D. R., and A. Y. Rudensky.** 2010. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell* **140:**845–858.
39. **Liu, Y., B. Yang, M. Zhou, L. Li, H. Zhou, J. Zhang, H. Chen, and C. Wu.** 2009. Memory IL-22-producing CD4+ T cells specific for *Candida albicans* are present in humans. *Eur. J. Immunol*. **39:**1472–1479.
40. **Ma, C. S., G. Y. Chew, N. Simpson, A. Priyadarshi, M. Wong, B. Grimbacher, D. A. Fulcher, S. G. Tangye, and M. C. Cook.** 2008. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. *J. Exp. Med*. **205:**1551–1557.
41. **Martinon, F., and J. Tschopp.** 2004. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* **117:**561–574.
42. **Martinon, F., and J. Tschopp.** 2005. NLRs join TLRs as innate sensors of pathogens. *Trends Immunol*. **26:**447–454.
43. **Mean, M., O. Marchetti, and T. Calandra.** 2008. Bench-to-bedside review: *Candida* infections in the intensive care unit. *Crit. Care* **12:**204.
44. **Mellor, A. L., and D. H. Munn.** 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol*. **4:**762–774.
45. **Milner, J. D., J. M. Brenchley, A. Laurence, A. F. Freeman, B. J. Hill, K. M. Elias, Y. Kanno, C. Spalding, H. Z. Elloumi, M. L. Paulson, J. Davis, A. Hsu, A. I. Asher, J. O'Shea, S. M. Holland, W. E. Paul, and D. C. Douek.** 2008. Impaired T_H17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* **452:** 773–776.
46. **Miossec, P., T. Korn, and V. K. Kuchroo.** 2009. Interleukin-17 and type 17 helper T cells. *N. Engl. J. Med*. **361:**888–898.
47. **Miyazato, A., K. Nakamura, N. Yamamoto, H. M. Mora-Montes, M. Tanaka, Y. Abe, D. Tanno, K. Inden, X. Gang, K. Ishii, K. Takeda, S. Akira, S. Saijo, Y. Iwakura, Y. Adachi, N. Ohno, K. Mitsutake, N. A. Gow, M. Kaku, and K. Kawakami.** 2009. Toll-like receptor 9-dependent activation of myeloid dendritic cells by deoxynucleic acids from *Candida albicans*. *Infect. Immun*. **77:**3056–3064.
48. **Moretti, S., S. Bellocchio, P. Bonifazi, S. Bozza, T. Zelante, F. Bistoni, and L. Romani.** 2008. The contribution of PARs to inflammation and immunity to fungi. *Mucosal Immunol*. **1:**156–168.
49. **Netea, M. G., G. D. Brown, B. J. Kullberg, and N. A. Gow.** 2008. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat. Rev. Microbiol*. **6:**67–78.
50. **Netea, M. G., N. A. Gow, C. A. Munro, S. Bates, C. Collins, G. Ferwerda, R. P. Hobson, G. Bertram, H. B. Hughes, T. Jansen, L. Jacobs, E. T. Buurman, K. Gijzen, D. L. Williams, R. Torensma, A. McKinnon, D. M. MacCallum, F. C. Odds, J. W. Van der Meer, A. J. Brown, and B. J. Kullberg.** 2006. Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Investig*. **116:**1642–1650.
51. **Netea, M. G., B. J. Kullberg, and J. W. van der Meer.** 2005. Severely impaired IL-12/IL-18/IFNγ axis in patients with hyper IgE syndrome. *Eur. J. Clin. Investig*. **35:** 718–721.
52. **Netea, M. G., R. Sutmuller, C. Hermann, C. A. Van der Graaf, J. W. Van der Meer, J. H. van Krieken, T. Hartung, G. Adema, and B. J. Kullberg.** 2004. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. *J. Immunol*. **172:**3712–3718.
53. **Odds, F. C., and M. D. Jacobsen.** 2008. Multilocus sequence typing of pathogenic *Candida* species. *Eukaryot. Cell* **7:**1075–1084.
54. **Orabona, C., U. Grohmann, M. L. Belladonna, F. Fallarino, C. Vacca, R. Bianchi, S. Bozza, C. Volpi, B. L. Salomon, M. C. Fioretti, L. Romani, and P. Puccetti.** 2004. CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. *Nat. Immunol*. **5:**1134–1142.
55. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev*. **20:**133–163.
56. **Pirofski, L. A., and A. Casadevall.** 2006. Acquired antibody-mediated immunity to fungi, p. 487–503. *In* J. Heitman, S. G. Filler, J. E. Edwards, and A. P. Mitchell (ed.), *Molecular Principles of Fungal Pathogenesis*. ASM Press, Washington, DC.
57. **Reid, D. M., N. A. Gow, and G. D. Brown.** 2009. Pattern recognition: recent insights from Dectin-1. *Curr. Opin. Immunol*. **21:**30–37.
58. **Rizzetto, L., M. Kuka, C. De Filippo, A. Cambi, M. G. Netea, L. Beltrame, G. Napolitani, M. G. Torcia, U. D'Oro, and D. Cavalieri.** 2010. Differential IL-17 production and mannan recognition contribute to fungal pathogenicity and commensalism. *J. Immunol*. **184:**4258–4268.
59. **Robinson, M. J., F. Osorio, M. Rosas, R. P. Freitas, E. Schweighoffer, O. Gross, J. S. Verbeek, J. Ruland, V. Tybulewicz, G. D. Brown, L. F. Moita, P. R. Taylor, and C. Reis e Sousa.** 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med*. **206:**2037–2051.
60. **Romagnani, S., E. Maggi, F. Liotta, L. Cosmi, and F. Annunziato.** 2009. Properties and origin of human Th17 cells. *Mol. Immunol*. **47:**3–7.
61. **Romani, L.** 2011. Immunity to fungal infections. *Nat. Rev. Immunol*. 11:275–288.
62. **Romani, L.** 2004. Immunity to fungal infections. *Nat. Rev. Immunol*. **4:**1–23.
63. **Romani, L., F. Bistoni, and P. Puccetti.** 2002. Fungi, dendritic cells and receptors: a host perspective of fungal virulence. *Trends Microbiol*. **10:**508–514.
64. **Romani, L., C. Montagnoli, S. Bozza, K. Perruccio, A. Spreca, P. Allavena, S. Verbeek, R. A. Calderone, F. Bistoni, and P. Puccetti.** 2004. The exploitation of distinct recognition receptors in dendritic cells determines the full range of host immune relationships with *Candida albicans*. *Int. Immunol*. **16:**149–161.
65. **Romani, L., and P. Puccetti.** 2006. Protective tolerance to fungi: the role of IL-10 and tryptophan catabolism. *Trends Microbiol*. **14:**183–189.
66. **Romani, L., T. Zelante, A. De Luca, F. Fallarino, and P. Puccetti.** 2008. IL-17 and therapeutic kynurenines in pathogenic inflammation to fungi. *J. Immunol*. **180:** 5157–5162.
67. **Ruas, L. P., E. S. Bernardes, M. L. Fermino, L. L. de Oliveira, D. K. Hsu, F. T. Liu, R. Chammas, and M. C. Roque-Barreira.** 2009. Lack of galectin-3 drives response to *Paracoccidioides brasiliensis* toward a Th2-biased immunity. *PLoS One* **4:**e4519.
68. **Ryan, K. R., M. Hong, P. D. Arkwright, A. R. Gennery, C. Costigan, M. Dominguez, D. Denning, V. McConnell, A. J. Cant, M. Abinun, G. P. Spickett, and D. Lilic.** 2008. Impaired dendritic cell maturation and cytokine production in patients with chronic mucocutanous [sic] candidiasis with or without APECED. *Clin. Exp. Immunol*. **154:**406–414.
69. **Ryan, K. R., C. A. Lawson, A. R. Lorenzi, P. D. Arkwright, J. D. Isaacs, and D. Lilic.** 2005. CD4 +CD25+ T-regulatory cells are decreased in patients with autoimmune polyendocrinopathy candidiasis ectodermal dystrophy. *J. Allergy Clin. Immunol*. **116:**1158–1159.

70. **Saijo, S., S. Ikeda, K. Yamabe, S. Kakuta, H. Ishigame, A. Akitsu, N. Fujikado, T. Kusaka, S. Kubo, S. H. Chung, R. Komatsu, N. Miura, Y. Adachi, N. Ohno, K. Shibuya, N. Yamamoto, K. Kawakami, S. Yamasaki, T. Saito, S. Akira, and Y. Iwakura.** 2010. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against *Candida albicans*. *Immunity* **32:**681–691.
71. **Sato, K., X. L. Yang, T. Yudate, J. S. Chung, J. Wu, K. Luby-Phelps, R. P. Kimberly, D. Underhill, P. D. Cruz, Jr., and K. Ariizumi.** 2006. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J. Biol. Chem.* **281:**38854–38866.
72. **Schneider, D. S., and J. S. Ayres.** 2008. Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. *Nat. Rev. Immunol.* **8:**889–895.
73. **Sganga, G.** 2009. Clinical aspects of invasive candidiasis in the surgical patient. *Drugs* **69(**Suppl. 1)**:**29–32.
74. **Singh, N., and J. R. Perfect.** 2007. Immune reconstitution syndrome associated with opportunistic mycoses. *Lancet Infect. Dis.* **7:**395–401.
75. **Sundrud, M. S., S. B. Koralov, M. Feuerer, D. P. Calado, A. E. Kozhaya, A. Rhule-Smith, R. E. Lefebvre, D. Unutmaz, R. Mazitschek, H. Waldner, M. Whitman, T. Keller, and A. Rao.** 2009. Halofuginone inhibits TH17 cell differentiation by activating the amino acid starvation response. *Science* **324:**1334–1338.
76. **Talloczy, Z., J. Martinez, D. Joset, Y. Ray, A. Gacser, S. Toussi, N. Mizushima, J. D. Nosanchuk, H. Goldstein, J. Loike, D. Sulzer, and L. Santambrogio.** 2008. Methamphetamine inhibits antigen processing, presentation, and phagocytosis. *PLoS Pathog.* **4:**e28.
77. **Taylor, P. R., S. V. Tsoni, J. A. Willment, K. M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G. D. Brown.** 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* **8:**31–38.
78. **van de Veerdonk, F. L., R. J. Marijnissen, B. J. Kullberg, H. J. Koenen, S. C. Cheng, I. Joosten, W. B. van den Berg, D. L. Williams, J. W. van der Meer, L. A. Joosten, and M. G. Netea.** 2009. The macrophage mannose receptor induces IL-17 in response to *Candida albicans*. *Cell Host Microbe* **5:**329–340.
79. **van de Veerdonk, F. L., M. G. Netea, L. A. Joosten, J. W. van der Meer, and B. J. Kullberg.** 2010. Novel strategies for the prevention and treatment of *Candida* infections: the potential of immunotherapy. *FEMS Microbiol Rev.* **34:**1063–1075.
80. **van Spriel, A. B., M. Sofi, K. H. Gartlan, A. van der Schaaf, I. Verschueren, R. Torensma, R. A. Raymakers, B. E. Loveland, M. G. Netea, G. J. Adema, M. D. Wright, and C. G. Figdor.** 2009. The tetraspanin protein CD37 regulates IgA responses and anti-fungal immunity. *PLoS Pathog.* **5:**e1000338.
81. **Venditti, M.** 2009. Clinical aspects of invasive candidiasis: endocarditis and other localized infections. *Drugs* **69** (Suppl. 1)**:**39–43.
82. **Villamon, E., D. Gozalbo, P. Roig, J. E. O'Connor, D. Fradelizi, and M. L. Gil.** 2004. Toll-like receptor-2 is essential in murine defenses against *Candida albicans* infections. *Microbes Infect.* **6:**1–7.
83. **Wells, C. A., J. A. Salvage-Jones, X. Li, K. Hitchens, S. Butcher, R. Z. Murray, A. G. Beckhouse, Y. L. Lo, S. Manzanero, C. Cobbold, K. Schroder, B. Ma, S. Orr, L. Stewart, D. Lebus, P. Sobieszczuk, D. A. Hume, J. Stow, H. Blanchard, and R. B. Ashman.** 2008. The macrophage-inducible C-type lectin, Mincle, is an essential component of the innate immune response to *Candida albicans*. *J. Immunol.* **180:**7404–7413.
84. **Yamasaki, S., E. Ishikawa, M. Sakuma, H. Hara, K. Ogata, and T. Saito.** 2008. Mincle is an ITAM-coupled activating receptor that senses damaged cells. *Nat. Immunol.* **9:**1179–1188.
85. **Yamasaki, S., M. Matsumoto, O. Takeuchi, T. Matsuzawa, E. Ishikawa, M. Sakuma, H. Tateno, J. Uno, J. Hirabayashi, Y. Mikami, K. Takeda, S. Akira, and T. Saito.** 2009. C-type lectin Mincle is an activating receptor for pathogenic fungus, *Malassezia*. *Proc. Natl. Acad. Sci. USA* **106:**1897–1902.
86. **Zelante, T., A. De Luca, P. Bonifazi, C. Montagnoli, S. Bozza, S. Moretti, M. L. Belladonna, C. Vacca, C. Conte, P. Mosci, F. Bistoni, P. Puccetti, R. A. Kastelein, M. Kopf, and L. Romani.** 2007. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur. J. Immunol.* **37:**2695–2706.
87. **Zelante, T., A. De Luca, C. D'Angelo, S. Moretti, and L. Romani.** 2009. IL-17/Th17 in anti-fungal immunity: what's new? *Eur. J. Immunol.* **39:**645–648.
88. **Zelante, T., F. Fallarino, F. Bistoni, P. Puccetti, and L. Romani.** 2009. Indoleamine 2,3-dioxygenase in infection: the paradox of an evasive strategy that benefits the host. *Microbes Infect.* **11:**133–141.
89. **Zenewicz, L. A., and R. A. Flavell.** 2008. IL-22 and inflammation: leukin' through a glass onion. *Eur. J. Immunol.* **38:**3265–3268.
90. **Zhou, L., M. M. Chong, and D. R. Littman.** 2009. Plasticity of CD4+ T cell lineage differentiation. *Immunity* **30:**646–655.

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch10

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Mucosal Immunity to *Candida albicans*

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INTRODUCTION AND HISTORICAL PERSPECTIVE

Prior to the human immunodeficiency virus (HIV) epidemic, host defense against *Candida albicans* at mucosal sites was largely considered one dimensional. Although general anatomical distinctions were always understood relative to immunity and host defense, a concept that "all mucosae are equal" was often implied for mucosal candidiasis, whereby discussions of host defense at one mucosal site were generally applied to other mucosal sites as well. This was primarily due to the lack of large populations of individuals with a specific type of mucosal candidiasis in any one geographical location. Oral candidiasis was relatively uncommon until transplantation and other forms of therapeutic immunosuppression became more prevalent. Despite the resulting increases in oral infections, the numbers of patients available for clinical studies at any one location remained quite small. Vaginal candidiasis has always been prevalent, but it had never been taken very seriously as a mucosal fungal infection due to the anecdotal nature of reports on causes and treatment regimens. Gastrointestinal (GI) tract infections are difficult to diagnose and usually not studied in humans. These issues hindered the ability to perform large-scale cohort studies of site-specific mucosal infections.

Prior to in-depth analyses of specific mucosal sites, the majority of mucosal *Candida* infections were thought to be associated with some form of immunoglobulin A (IgA) antibody or humoral immunodeficiency. However, when antibody deficiency was difficult to demonstrate (116, 146, 163), it was the syndrome of chronic mucocutaneous candidiasis that stimulated the next series of explanations. Chronic mucocutaneous candidiasis is characterized by chronic infections of mucosal surfaces, skin, and nails and was considered to be due to a deficiency in peripheral (blood) T-cell-mediated immunity (114–117). Therefore, all other mucosal *Candida* infections were also considered to be caused by a similar T-cell deficiency despite their tendencies to be site specific and not multifocal. It was not until the HIV epidemic that clear distinctions in mucosal responses to infection became apparent.

The HIV epidemic created large numbers of individuals with severe immunosuppression and resulted in a significant increase in oral candidiasis. Accordingly, "mucosal candidiasis" became a means to refer predominantly to oropharyngeal candidiasis (OPC) or esophageal candidiasis because these were the most prevalent manifestations of HIV infection. In fact, esophageal candidiasis has been considered an AIDS-defining illness for much of the epidemic period (118, 138, 169). Although vulvovaginal candidiasis (VVC) was a possible AIDS-defining illness at one point (27, 67, 203), it soon became clear that vaginal candidiasis was really no more common in HIV-positive (HIV^+) than in HIV^- women (44, 102, 129, 179, 192, 227). Following immunological studies of vaginal candidiasis (reviewed in reference 84) and in HIV-infected persons with OPC (reviewed in reference 74), the physiological anatomical distinctions became more evident and supported the concept that host defenses against *Candida* at the oral and vaginal mucosae were unique, distinct, and independent. In addition, the vast amount of data accumulated over the past 20 years showing distinct T-cell subpopulations, antigen-presenting cells (i.e., dendritic cells [DCs], macrophages, and Langerhans cells), and B cells at different mucosal sites have confirmed that mucosal sites are independent and unique relative to host immunoreactivity (85, 98, 100, 103, 104, 109, 154). As a result, it is now accepted that all mucosae are *not* equal in terms of *Candida* infections and that host defense at the various mucosal sites needs to be studied independently and exclusively at the local level with respect to cells and soluble immune factors.

This chapter is divided into in-depth reviews of host defense mechanisms against oral, vaginal, and GI candidiasis, with emphasis on the uniqueness of the responses at each site. We provide a historical perspective of research in each area, followed by an overview of current studies. In comparing the responses at these sites, there is significant evidence to support the idea that host defense against OPC is dependent on cell-mediated immunity (CMI) and $CD4^+$ Th1-type responses, yet other nonconventional host defenses are also critically important (74, 126, 242). On the other hand, natural host defense against VVC does not appear to include T cells or any type of adaptive immunity, but instead relies on innate immunity for both protection and susceptibility to

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infection (75, 76). Defense against GI candidiasis involves both intact barrier function and innate and adaptive immunity to prevent mucosal infection and dissemination, which can be a clinically significant outcome of this type of infection. We follow with a short discussion of the emerging role of mucosal *Candida* biofilms in pathogenesis and host defense. The chapter concludes with suggested future directions for the field of mucosal immunity and candidiasis.

HOST DEFENSE AGAINST ORAL CANDIDIASIS

Oropharyngeal Candidiasis

OPC encompasses infections of the hard and soft palate, tongue, buccal mucosa, and floor of the mouth and can present as reddened patches (erythematous) or white curd-like lesions (pseudomembranous). Chewing and swallowing can be difficult and painful under these conditions. Infections can be acute or recurrent and are common in immunocompromised patients. Although OPC occurs with several immunocompromising conditions, it appears to be much more common in HIV-infected persons than under any other condition (91, 159, 187, 191). In fact, OPC is often one of the first clinical signs of underlying HIV infection and occurs in 50 to 95% of all HIV-positive persons sometime during their progression to AIDS (172). Thus, it is possible that a link with HIV is present and enhances susceptibility to OPC. Interestingly, highly active antiretroviral therapy (HAART) has reduced the incidence of OPC (166, 213). This reduction is postulated to be due both to increased immune responsiveness and to the action of the protease inhibitors in HAART on the secretory aspartyl proteases (Saps), important virulence factors for *C. albicans* (29, 37, 92).

No Role for Humoral Immunity

Early studies of *Candida*-specific antibodies in saliva of HIV-positive patients with or without OPC found similar or elevated levels of IgA or IgG (47), although a reduced affinity of *Candida*-specific IgA antibodies was reported for patients with AIDS. There is no evidence to date, however, that a deficiency in *Candida*-specific antibodies is present in HIV$^+$ patients that could account for the increased prevalence of OPC (38, 151, 235, 238). Therefore, humoral immunity does not appear to play a role in protection against or susceptibility to OPC.

A Major Role for CD4$^+$ T Cells

Studies to date suggest that CD4$^+$ Th1-type cells are the most critical host defense against OPC. Clinically, OPC is most common in HIV$^+$ patients when CD4$^+$ cell numbers drop below 200 cells/μl (91, 159, 172, 191). In vitro immune analyses demonstrated that peripheral blood mononuclear cells (PBMC) from most individuals respond to *Candida* antigens with Th1-type cytokines. Thus, it was generally considered that the increased susceptibility to OPC during CD4$^+$ deficiency was due to either a lack of protective Th1-type responses or a shift to susceptible Th2-type responses (182). Some early studies with HIV$^+$ individuals showed a strong association between OPC and reduced Th1-type cytokine responses (171). However, several subsequent studies evaluating PBMC reactivity in HIV$^-$ and HIV$^+$ individuals with or without symptomatic OPC stratified by CD4$^+$ T cell number showed little to no appreciable differences in *Candida*-specific proliferation or cytokine production between the two groups (129, 164). In another study, no demonstrable deficiencies in clonal responses of PBMC from HIV$^+$ patients to a variety of antigenic peptides were shown (124). Together, these results suggested not that the *Candida*-specific T cells themselves become defective as a result of HIV infection but, rather, that a threshold number of CD4$^+$ T cells is required to protect the oral cavity against infection by this commensal organism. Below this threshold number of cells, other systemic or local immune mechanisms would need to function if available. The prevalence of OPC, then, depends on the status of the local immune mechanisms. Indeed, some individuals with <200 CD4$^+$ cells/μl never have OPC, while others have recurrent episodes of OPC.

Several experimental mouse models also support a role for a combination of T cells and innate cells against OPC, as well as specific roles for CD8$^+$ T cells. In these models, both CD4$^+$ and CD8$^+$ T cells along with macrophages were recruited into the mucosal tissue following oral inoculation, and intraepithelial CD4$^+$ T cells persisted after resolution of the infection (2, 41). Moreover, there was also a time-dependent recruitment of γ/δ TCR$^+$ cells that correlated with the resolution of the disease (41, 68). Analysis of cytokines showed a role for both Th1- and Th2-type cytokines in resistance to infection depending on the strain of mice used (68). Another study examined cytokines in oral tissues from infected mice and found increases in interleukin 6 (IL-6), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) in mice recovering from oral infection (71). Interestingly, no IL-2, IL-4, or IL-10 was detected during infection (71). As with other forms of mucosal candidiasis, however, little is known concerning the specific roles of local versus systemic T cells in protection against infection.

Experimental animal studies have yielded additional information on the requirement for CD4$^+$ T cells in defense against OPC. In a study employing in vivo cellular depletions to create immunocompromised mice, depletion of CD4$^+$ T cells alone did not increase severity of disease (70). However, a combination of head and neck irradiation with CD4$^+$ T-cell depletion led to persistent oral infection. Interestingly, a murine AIDS model showed a rate of 30% recurrent OPC in inoculated mice, with a predominance of CD8$^+$ T cells recruited into the tissues (58). In addition, CD4C/HIV(mut) transgenic mice that express *nef*, *env*, and *rev* genes from HIV and lack CD4$^+$ T cells were highly susceptible to OPC (57). In studies evaluating host defense factors associated with infection, CD8$^+$ T cells were shown to be present in the oral tissue, although no mention of cellular location was made (90, 145). Nevertheless, the latter models are likely to reflect the clinical situation in the HIV$^+$ patient.

Local Immunity in the Immunocompromised: Absence of CD4$^+$ T Cells

Several aspects of local immunity have been evaluated clinically in OPC. In support of the Th1/Th2 dichotomy concept, it was reported that HIV$^-$ individuals had Th1/Th0 cytokines in their saliva, whereas HIV$^+$ individuals had primarily Th2-type cytokines, which were more profound in those patients with OPC (132). Interestingly, the Th2-type profile was the result of reduced Th1-type cytokines rather than increased Th2-type cytokines. Lymphocytes have also been examined in OPC lesions, and both CD4$^+$ and CD8$^+$ cells have been identified (180). However, other studies have shown that only CD8$^+$ cells were present and that they accumulated at a considerable distance from the fungal in-

fection, which is localized superficially at the outer epithelium (153). This cellular accumulation is not seen in OPC$^-$ persons. This suggested a role for CD8$^+$ T cells against infection, with a potential problem in cell trafficking or the microenvironment, thereby promoting susceptibility to OPC. In support of the presence of activated CD8$^+$ T cells, mRNA for several CD8 cell-associated cytokines (IL-2 and IL-15) and chemokines (IP-10, RANTES, and MCP-1) was found to be increased in HIV$^+$ patients with OPC compared to those with asymptomatic *Candida* colonization (133, 134).

Role of CD8$^+$ T Cells in Local Responses

In characterizing tissue-associated CD8$^+$ T cells, immunohistochemical analysis showed that the majority of the cells possess αβ T-cell receptors (147). Regarding the local immune environment and immune status of the tissue-associated CD8$^+$ T cells, results showed that they were normal activated memory T cells (CD69$^+$ CD45RO$^+$) as a mix of effector (CD62L$^-$ CCR7$^-$) and central (CD27$^+$) memory cells. Furthermore, the CD8$^+$ T cells were not considered to be natural killer (NK) T cells or anti-HIV CD8$^+$ T cells (30, 59, 101). In parallel studies, homing receptors (e.g., $\alpha_4\beta_7$, $\alpha_4\beta_1$, and $\alpha_e\beta_7$) that govern the migration of cells through interactions with tissue adhesion molecules (VCAM-1, MAdCAM, and E-cadherin) were also normally expressed on the CD8$^+$ T cells in those with OPC. Together, the results suggested that the accumulated mucosal migration-challenged CD8$^+$ T cells are otherwise normal memory T cells in an activated state (130). In contrast, differences in tissue-associated adhesion molecule expression were detected. MAdCAM expression was significantly increased in OPC$^+$ tissue, in support of the increased presence of T cells. E-cadherin, on the other hand, was significantly decreased in OPC$^+$ tissue. Interestingly, the expression of E-cadherin was localized to the epithelium, which is critical for migration of cells to the outer epithelium, where the *Candida* infection (lesion) is primarily located. Therefore, it was suggested that the decrease in E-cadherin may limit the ability of the CD8$^+$ T cells to migrate to the outer epithelium, representing a possible dysfunction in those with OPC. Currently, the underlying hypothesis for susceptibility to OPC in HIV$^+$ persons with reduced CD4$^+$ T cells is that those protected against OPC have normal levels of E-cadherin and CD8$^+$ T cells are capable of migrating to the outer epithelium. On the other hand, in those with OPC, E-cadherin is suboptimal, resulting in lack of migration by the CD8$^+$ T cells and risk for OPC.

Interestingly, results of recent longitudinal analyses conducted with patients with a history of OPC showed that the reduced E-cadherin expression is not permanent (Fidel and coworkers, unpublished data). These observations are supported by two independent studies showing that *Candida* can cleave or degrade E-cadherin (87, 219). Hence, the reduction in E-cadherin may be a virulence mechanism for *Candida* that promotes adherence to the epithelium and subsequent invasion. Hence, the differential E-cadherin expression may fluctuate with *Candida* levels. This lends to the further hypothesis that increases in *Candida* levels lead to increased degradation of E-cadherin, which creates an environment more conducive to infection and invasion and the onset of OPC. If so, immunotherapeutic strategies directed towards restoring E-cadherin expression would allow CD8$^+$ T cells to migrate to the outer epithelium, where they could exert antifungal effector activity, thereby reducing the incidence of OPC in susceptible populations.

Innate Immunity

Role for Epithelial Cells

Epithelial cells represent another level of local immunity. Oral epithelial cells have been shown to inhibit up to 80% of the growth of *Candida* species in vitro by a static mechanism via cell contact, with no role for soluble factors (161, 205, 207). Analysis of oral epithelial cells in HIV$^+$ individuals showed significantly reduced activity in cells from patients with OPC compared to those from patients without OPC, providing evidence for an innate form of protection (205). Additionally, epithelial cells produce both cytokines and chemokines in response to *Candida*, which may contribute to the innate and/or adaptive immune response (63, 64, 183, 189, 204). Studies evaluating the properties of the effector moiety revealed it to be an acid-labile protein (242) that did not require any demonstrable metabolic activity by the epithelial cells for the effector function (206). Thus, the activity was dependent on intact, but not necessarily live, epithelial cells. Further studies showed that extraction of surface proteins from the cells exhibited antifungal activity similar to that of intact epithelial cells (135). Using the acid-labile property as a tool together with proteomic analyses and functional assays, the acid-labile protein was identified as 33-kDa annexin A1 (135). Annexin A1 represents a viable candidate for the effector molecule, as it functions through signaling cascades to inhibit cellular processes, including growth (49, 136).

Role of Neutrophils

Polymorphonuclear cells (PMNs) are considered to play a role in innate defenses against OPC because neutropenic cancer patients are susceptible to disease (3). However, data from experimental models are controversial. In a non-HIV murine model, a combination of PMN depletion and macrophage inactivation via carrageenan treatment resulted in exacerbated oral infection (70). On the other hand, studies using the HIV transgenic mice described above showed that PMNs functioned normally in these mice and that depletion had no effect on oral fungal burdens in inoculated animals (145). While obviously more studies need to be conducted, it is possible that PMNs work in concert with other facets of host defense during HIV-associated OPC, including other types of phagocytes.

More recent studies point to a role for the Th17 pathway, which modulates PMN function, in protection against OPC. Th17 cells are a recently discovered type of effector CD4$^+$ T cell with a distinct lineage and cytokine profile compared with those of Th1 and Th2 cells. Naïve T cells differentiate into Th17 cells in the presence of a combination of transforming growth factor β (TGF-β), IL-6, IL-1, and IL-21, while IL-23 is essential for Th17 cell expansion and function. Th17 cells secrete a unique profile of cytokines, including IL-17 (IL-17A), IL-17F, IL-21, and IL-22 (reviewed in reference 122). Production of these cytokines induces neutrophil-activating factors, antimicrobial peptides, and acute-response proteins (reviewed in reference 4). Interestingly, knockout mice lacking IL-23 or IL-17R (Th17 deficient) were highly susceptible to OPC, whereas mice lacking IL-12 (Th1 deficient) were relatively resistant to infection (46). This result may seem contrary to earlier studies that showed that CD4$^+$ T-cell deficiency alone does not lead to increased susceptibility to experimental OPC (70). However, a large number of non-CD4$^+$ cells secrete and respond to Th17 cytokines. These "Th17-like" cells include CD4$^-$ CD8$^-$ and CD4$^-$ CD8$^+$ T cells, γδ T cells, iNKT cells,

NK cells, LTi, and possibly myeloid cells. Therefore, the Th17 response may be relatively intact in $CD4^+$ T-cell-deficient animals. Interestingly, HIV infection seems to preferentially target Th17 cells, as HIV^+ patients have reduced Th17 cells regardless of viral load or CD4 cell count (69). Therefore, HIV infection may predispose to OPC in part due to effects on the Th17 pathway, and this becomes exacerbated further as $CD4^+$ T cells are lost.

Role of Antimicrobial Peptides

Another mechanism of innate antifungal immunity in the oral cavity is production of antimicrobial peptides in saliva, which include calprotectin, β-defensins, and histatins. Human β-defensin 1 (hBD-1), hBD-2, and hBD-3 all have activity against *Candida* species, with hBD-2 and hBD-3 being most effective (72). They are fungicidal either through disruption of the fungal membrane or in an energy-dependent and salt-sensitive manner (72, 220). Histatin-5 is a cationic peptide that acts on the fungal membrane to release ATP and together with salt sensitivity and ion selectivity potentiates killing (221). Interestingly, hBDs and histatin-5 are additive in their antifungal effects (220). In a recent study, salivary histatin-5 was reported to be reduced in HIV^+ patients (214), and this reduction was proposed to be a factor in the increased prevalence of oral *Candida* colonization and OPC. A follow-up study reported that *C. albicans* Sap9 was capable of proteolytic cleavage of histatin-5, lending to a novel immune evasion strategy of *Candida* and an explanation for the reduced levels of histatin-5 in HIV disease (148).

Defense against Oral Dissemination

Disseminated disease is not a common sequela of OPC, even in immunocompromised individuals. A recent study demonstrated a role for the proinflammatory cytokine IL-1β in host defense against OPC using a murine model in which oral tissues were scraped to damage the epitlhelial barrier defenses. Interestingly, data also suggested that IL-1β has an important role in resistance to extraoral dissemination of *Candida*. Depletion of IL-1β in mice led to increased oral colonization and dissemination of *Candida* to extraoral sites concomitant with significant mortality (97). *C. albicans* induces IL-1β transcription through pattern recognition receptors (PRRs) Toll-like receptor 2 (TLR2) and dectin-1 on host cells, and processing of IL-1β is mediated by a larger intracellular proinflammatory multiprotein complex termed the NLRP3 inflammasome (97).

Conclusion: Multilayered-Defense Hypothesis

Several lines of defense may be important for protection against OPC, many of which do not become evident until $CD4^+$ cells are reduced below the protective threshold. Accordingly, it has been proposed that there are both primary and secondary host defenses that protect against OPC. The primary host defenses are the $CD4^+$ T cells that are maintained at a threshold number and are recruited into the oral mucosa when necessary. Th1-type cytokines in saliva may also contribute to protection. Secondary host defenses include the $CD8^+$ T cells and other innate cells such as PMNs, macrophages, and epithelial cells, as well as antimicrobial peptides. The origin of the $CD8^+$ T cells as well as the mechanism of action of antifungal activity is currently unknown. Since standard cytotoxic T-lymphocyte activity by $CD8^+$ T cells is not active against fungal organisms, it is speculated that it may be a non-HLA-restricted growth inhibition activity that has been described for murine cells (18, 19) and for humans against HIV (139, 209). There may be a new paradigm for host defense against OPC that involves a primary role for Th17 responses as an innate resistance mechanism that may function in concert with the adaptive Th1-type responses as well as $CD8^+$ T cells to ultimately keep *Candida* in a commensal state.

Denture Stomatitis

Another form of oral candidiasis is *Candida*-associated denture stomatitis (DS). DS is the most prevalent form of oral candidiasis, affecting up to 65% of otherwise healthy denture wearers (26, 51). DS is characterized by an inflamed oral mucosa, halitosis, and burning or bleeding of the infected mucosa (reviewed in reference 223). *Candida albicans* is the most common cause of DS and can readily form biofilms on denture material. Research on the pathogenesis of DS was most formidable in the 1970s and 1980s and employed several animal models (165, 194). However, host response was not avidly studied, as much of contemporary immunology was in its infancy. Since the 1980s, research on DS has been limited, presumably due to the focus on the HIV epidemic, in which OPC became extremely common as an AIDS-defining illness. Few clinical studies have been conducted except for those associated with etiology. In fact, patients with DS were often used as controls for HIV^+ cohorts since DS is a form of oral candidiasis in an immunocompetent population. The lone study that evaluated immune reactivity showed that, as expected, patients with DS were not deficient in Th1-type salivary cytokines as HIV^+ OPC^+ patients were (131). But as discussed later in this chapter, research in DS is becoming more common again, with studies to evaluate the role of biofilm and host defense in the pathogenesis of disease through the use of contemporary research tools.

HOST DEFENSE AGAINST GI CANDIDIASIS

Candida species are normal inhabitants of the GI tract, with colonization rates ranging from 30 to 70% among healthy adults (119, 200). Mechanisms by which *Candida* persists at mucosal surfaces in the face of an adaptive immune response are relatively unknown. Persistence in the GI tract can lead to GI candidiasis (defined as infection of the stomach and small and large intestines). More importantly, GI colonization and infection predispose patients to systemic candidiasis due to outgrowth from the GI tract, which is also known as candidiasis of endogenous origin (141, 142, 195, 225). Currently, *Candida* species are the fourth leading cause of bloodstream infections in the United States, with many of these infections originating from the GI tract (176). *C. albicans* is the predominant species identified, although infections due to non-*C. albicans* species are on the rise (17, 52).

GI candidiasis is not easily recognized clinically due to the lack of specific symptoms and the lack of the ability to differentiate between pathogenic and commensal *C. albicans*. Unlike with OPC and VVC, analysis of *Candida* morphology is not predictive of disease. In murine models of infection, hyphae are primarily observed in keratinized epithelium (stomach), but yeasts predominate on nonkeratinized epithelium, with ≈70% of yeasts present in the intestinal tract during infection (7, 228). However, it is clear from many human studies that two major predisposing factors are immunodeficiency and antibiotic therapy. Types of

patients at risk for GI candidiasis include cancer and transplant patients receiving immunosuppressive therapy, neutropenic patients, and patients receiving prolonged antibiotic prophylaxis (3, 186). Therefore, both the host immune response and a normal bacterial microbiota are involved in controlling commensalism versus disease.

Role of Innate Immunity

Most of what is known about innate host defense against GI candidiasis and dissemination has come from studies using gnotobiotic immunodeficient mice. In terms of innate immunity, mice with congenital granulocytic cell deficiencies (*bg/bg*) that affect macrophages, PMNs, and NK cells were susceptible to GI candidiasis but mounted *Candida*-specific T-cell and antibody responses and eventually cleared the infection (32). Treatment with a nitric oxide synthase inhibitor increased the severity of GI tract infection. However, the reactive species responsible is likely peroxynitrite, as nitric oxide is not directly candidacidal in vitro (218). Mice lacking phagocyte oxidase ($Phox^{-/-}$) and nitric oxide synthase 2 ($NOS2^{-/-}$) were susceptible not only to GI mucosal infection but also to dissemination and morbidity (13). These studies underscore the importance of oxidative pathways of granulocytes to defend against both mucosal infection and the ability to disseminate from the GI tract.

Role of Adaptive Immunity

Studies using congenitally athymic T-cell-deficient (*nu/nu*), severe combined immunodeficient (SCID; no T or B cells) mice, or mice specifically lacking α/β or γ/δ T cells have shown that T cells are critical for effective protection against GI candidiasis (7, 9, 31, 111). Mice with T-cell deficiencies were more susceptible to GI tract infection but were not susceptible to sublethal intravenous challenge or dissemination from the GI tract. Wild-type mice cleared the infection and responded to *Candida* antigens with IL-2 production and delayed-type hypersensitivity reactions, while T-cell-deficient mice did not. Likewise, antibody-mediated depletion of $CD4^+$ T cells increased the severity of GI tract infection but did not lead to dissemination. Further studies showed that both Th1 and Th2 cytokines were produced by mesenteric and Peyer's patch $CD4^+$ T cells at a time corresponding to clearance of a *Candida* GI tract infection (40). Treatment with recombinant IL-4 receptor, which favored Th1 cell responses, promoted clearance, indicating some role for Th1 responses in controlling GI infection. Thus, GI candidiasis appears to conform to the Th1/Th2-type immune response pattern seen in oral candidiasis, namely, that Th1-type responses are associated with resistance against infection, while Th2-type responses promote susceptibility by being nonprotective (28, 39, 140, 182, 211, 239).

Linking Innate and Adaptive Immunity

The most recent data on host defense against GI candidiasis involve understanding the role of DCs in directing both local and systemic adaptive immunity. DCs have both phagocytic and antifungal activities and act as antigen-presenting cells to stimulate T-cell responses (158). GI DCs can directly access microbes in the lumen and differentiate microbes based on pathogen-associated molecular patterns and PRR signaling (177, 178). Isolated Peyer's patch DCs respond differentially to yeast cells and hyphae of *C. albicans* (25), indicating differential PRR interactions. While blastoconidia promote differentiation of inflammatory DCs, hyphae promote differentiation of tolerogenic DCs. Inflammatory DCs were characterized by production of Th17/Th2 cytokines via the MyD88 (myeloid differentiation primary response gene 88) pathway, whereas tolerogenic DCs produced Th1/Treg cytokines via the TRIF (Toll/IL-1 receptor domain containing adaptor inducing IFN-γ) signaling pathway. These tolerogenic DCs were able to ameliorate experimentally induced colitis in mice, suggesting that *C. albicans* can exert immunoregulatory activity as well as inflammatory responses, depending on morphology. However, the unique gene expression profile of *C. albicans* in vivo, which is a mixture of genes induced during various infections and stress conditions, may mean that yet another unique subset of DC develops in response to *Candida* in vivo. This is especially important considering the divergence in responses observed in vitro with respect to morphotype, as yeasts are the predominate morphotype in the GI tract but express hypha-specific genes under the control of regulators not observed in vitro (228).

Defects in Both Innate and Adaptive Immunity Lead to Dissemination

In terms of dissemination, only mice with combined deficiencies in both T cells and granulocytic cells (*bg/bg nu/nu*) succumbed to systemic infection of endogenous origin (33). In addition, β_2 $microglobulin^{-/-}$ mice, which lack major histocompatibility complex class I (MHCI) and are deficient in $CD4^+$ and $CD8^+$ T cells, NK cells, and FcRn-mediated phagocytosis, also developed disseminated candidiasis after oral challenge (10). To more specifically address the mechanisms involved in mediating resistance to dissemination, mice with T-cell deficiencies were treated with agents to selectively inhibit innate responses. SCID mice were administered anti-GR-1 antibody (depletes PMNs), carrageenan, or silica (impairs phagocytic cell function). All treatments enhanced susceptibility to disseminated infection in SCID mice (106, 107). On the other hand, SCID mice depleted of NK cells did not exhibit enhanced susceptibility (105). Mice lacking IL-8 receptor were also more susceptible to GI tract infection, which correlated with a slower influx of PMNs, which were found to be defective in antifungal activity in vitro (11). Poly(I · C), which is a TLR3 ligand and impairs macrophage antifungal activity, enhanced the susceptibility of SCID mice to systemic candidiasis of endogenous origin (105). Germfree transgenic epsilon 26 mice, which exhibit combined defects in T cells and NK lineage cells, were also more susceptible to GI tract infection but resisted disseminated infection for 2 weeks (12). Depletion of granulocytes abrogated this resistance to dissemination, suggesting that non-NK granulocytes aid in early defense against dissemination, while NK and T cells are required for sustained resistance to dissemination. In more recent studies, the role of specific components of innate and adaptive immunity along with mucosal damage was investigated in a model of disseminated infection of endogenous origin (120). While systemic chemotherapy with cyclophosphamide led to 100% mortality, selective neutrophil depletion, macrophage depletion, lymphopenia, or GI mucosal disruption with dextran sodium sulfate alone resulted in no mortality. However, a combination of mucosal disruption and neutrophil depletion led to GI tract translocation and disseminated infection with 100% mortality. Therefore, bypassing the first line of defense—barrier function provided by epithelial cells—is also a major risk factor for disseminated disease.

No Role for Humoral Immunity

The role of humoral immunity was evaluated because immunocompetent mice, which clear GI tract infections, also mounted a specific antibody response (IgG, IgA, and IgM) against *Candida*. Conversely, T-cell-deficient mice (*nu/nu*), which did not clear infection, also did not produce any IgG or IgA (8). However, mice deficient in B cells and antibody production (J_HD mice) were resistant to GI tract infection and systemic dissemination (10). In addition, while oral immunization of germfree wild-type mice elicited a Th1 and antibody response that protected against systemic challenge, immunization had no effect on GI tract colonization levels in mice (108). This indicates that humoral immunity does not play a role in preventing *C. albicans* GI colonization.

Immunoregulation and Tolerance

Immunoregulation in the GI tract involves active induction of tolerance to antigens, which is mediated by DC-driven induction of regulatory T cells (Tregs). Tregs limit development of inflammatory responses to innocuous antigens via both contact-dependent mechanisms and production of tolerogenic cytokines TGF-β and IL-10 (158). Mice deficient in either of the costimulatory molecules B7-2 and CD28 exhibited decreased GI colonization levels but increased immunopathology (152). This outcome was associated with decreased IL-10 production and $CD4^+$ $CD25^+$ Tregs. Mice lacking IL-10 also exhibit decreased fungal burden but suffer from immunopathological consequences of GI infection (56). Therefore, induction of Tregs during GI tract infection facilitates *Candida* colonization by downregulating inflammatory responses but limits pathology due to overexuberant inflammation via production of IL-10. That GI candidiasis is fairly rare may be due to a balance of innate and adaptive immunoreactivities that favors commensalism and tolerance.

Immunoregulatory responses were also required for resistance to reinfection systemically after GI challenge with *C. albicans*, indicating that local immune responses generated in the GI tract direct systemic immunity to *Candida*. Interestingly, any overexuberant response was not an uncontrolled Th1 response but instead was dependent on Th17 cytokines. While IL-12-deficient $p35^{-/-}$ mice ($Th17^+$) succumbed to GI tract infection and dissemination, IL-23-deficient $p19^{-/-}$ mice ($Th17^-$) were protected, with limited immunopathological inflammation (244). Neutrophils from IL-$23^{-/-}$ mice also had more potent antifungal effector functions, while addition of Th17 cytokines (IL17 and IL-23) inhibited PMN activity. This indicates that induction of a Th17 response impairs both clearance of infection via dysregulated PMN activity and a hyperinflammation, which can be exacerbated further in the absence of Tregs or IL-10.

Role of the Bacterial Microbiota

Another aspect that can influence host responses to GI tract infection is the bacterial microbiota, which contributes to resistance to colonization (168). Recent studies suggest that probiotic bacteria exert an antagonistic effect on *Candida* in the GI tract. Prior inoculation of germfree immunocompetent mice with probiotic bacteria (*Lactobacillus* and *Bifidobacterium*) reduced the incidence of infection and modulated *Candida*-specific antibody- and cell-mediated responses (222). In immunodeficient mice, prior probiotic colonization decreased the severity of mucosal infection and prolonged survival in *bg/bg nu/nu* mice. The possible mechanisms that contribute to this activity are direct anti-*Candida* activity by bacteria and/or immunomodulation. In humans, supplementation with probiotic formula significantly reduced *Candida* colonization in low-birth-weight neonates, who are at greater risk of disseminated candidiasis of endogenous origin (144). Therefore, probiotic therapy may be an effective preventive measure or adjunct to antifungal treatment in controlling *Candida* colonization levels in the GI tract.

Conclusion: Linking Experimental Models with Clinical Observations

Systemic candidiasis is a disease of immunocompromised patients, and the GI tract serves as a frequent source of disseminated infection (162). Therefore, factors leading to susceptibility to disseminated infection of endogenous origin are similar to those involved in protection from invasive infection in the GI tract (128). Similar to murine experimental models, multiple immune defects are associated with disseminated infection. In particular, cancer patients, transplant recipients, and neutropenic patients are all at greater risk (45). However, HIV^+ patients do not have increased rates of systemic candidiasis, indicating that $CD4^+$ T-cell deficiency alone is not enough to bypass defenses of the GI tract (190). In a murine model using HIV transgenic mice, there were also no differences in the ability of *C. albicans* to colonize or invade GI tract tissues (90). Therefore, a combination of multiple factors is required in defense against GI tract invasive infection and dissemination, including innate responses (neutrophils, macrophages, and epithelial barrier integrity) and adaptive responses (cell-mediated immunity). If one component is lacking, others will compensate and are sufficient for defense.

HOST DEFENSE AGAINST VAGINAL CANDIDIASIS

VVC affects a significant number of women, predominantly in their reproductive years (113, 196, 197). An estimated 75% of all women will experience an episode of acute VVC in their lifetime, with another 5 to 10% developing recurrent VVC (RVVC) (196, 197). Vulvovaginitis involves infections of the vaginal lumen as well as the vulva. Symptoms include burning, itching, soreness, abnormal discharge, and dyspareunia. Signs include vaginal and vulvar erythema and edema. Acute VVC has several known predisposing factors, including antibiotic and oral contraceptive usage, hormone replacement therapy, pregnancy, uncontrolled diabetes mellitus, and immunosuppressive therapy (113, 196, 197). RVVC is multifactorial in etiology but is usually defined as idiopathic, with no known predisposing factors in the majority of those affected (196, 197). In women with RVVC, antifungal therapy is highly effective for individual symptomatic attacks but does not prevent subsequent recurrence. There is little evidence that resistance to antifungal drugs plays a role in the pathogenesis of RVVC (137). Instead, susceptibility to RVVC is postulated to be immune based in that these otherwise healthy women experience repeated symptomatic episodes as a result of some immunological dysfunction or deficiency. Furthermore, recurrences are presumed to be a result of relapse rather than reinfection, since strain types of *C. albicans* tend to remain the same in women for multiple recurrences over several years (217). This observation is consistent with the fact that most antifungal

drugs are fungistatic rather than fungicidal and do not result in elimination of the infection.

No Role for Systemic or Local T Cells

The majority of early clinical studies tested women with RVVC and healthy women for systemic *Candida*-specific CMI (86, 99, 112, 149, 231, 232). Results were highly variable, with no consensus of any systemic immune factor missing in women with RVVC. As Th responses emerged to show that resistance to mucosal *Candida* infections was associated with a Th1-type response, whereas susceptibility to infection was associated with Th2-type responses (40, 181), comprehensive testing of women with RVVC showed no demonstrable deficiency in *Candida*-specific Th1-type CMI (81). The general consensus became that systemic immunity was not playing a dominant role at the vaginal mucosa and that any immunodeficiency in women with RVVC resided locally, although sporadic reports have challenged this view (48, 156). Nevertheless, the consensus was supported by several clinical observations, including that women with RVVC were not susceptible to other forms of mucocutaneous candidiasis, and women with HIV infection, who were susceptible to OPC, were no more susceptible to VVC than the healthy HIV^- population (84, 198).

Based on the controversies in the clinical studies, animal models were commonly employed to evaluate host defense mechanisms against vaginitis. While rodents are not colonized with *Candida* and thus have no existing immune sensitization to *Candida*, the use of estrogen could establish persistent colonization/infection to study host responses. What emerged was a tremendous amount of data that, as in the clinical studies, revealed a lack of any demonstrable role for systemic CMI against vaginitis (21, 23, 77, 80, 82, 83, 96). In effect, while *Candida*-specific Th1-type responses were induced in the systemic compartment (i.e., draining lymph nodes) of vaginally inoculated mice, reduced fungal burden could not be achieved. The exception was in an estrogen-dependent rat model of vaginitis that, in contrast to the mouse model, has a property of spontaneous resolution of fungal burden within a 3-week period (36, 55). In this model, T cells could be demonstrated to infiltrate the vaginal mucosa following infection and Th1-type cytokines were present in vaginal secretions (55). Additionally, adoptive transfer of both $CD4^+$ and $CD8^+$ T cells from previously infected rats accelerated clearance of *Candida* in the vaginae of naïve animals, with $CD4^+$ cells being more effective at clearance (188). The same rat model of vaginitis also showed a role for humoral immunity, as *Candida*-specific anti-mannan and anti-aspartyl IgA antibodies protected against vaginitis (36, 53). This was a peculiar observation, since there were few, if any, clinical data showing a role for humoral immunity against any form of mucosal candidiasis, and studies of natural *Candida*-specific antibodies in the vaginae of infected mice were essentially nonexistent (78, 116, 146, 237). However, these IgA antibodies could be "protective" based on the Casadevall concept of nonprotective, protective, and indifferent antibodies (35). In support of this, Han and coworkers have shown that two different "protective" antibodies specific for mannan (IgM and IgG3) protect mice against *Candida* vaginitis when given either locally or systemically (93, 94).

Other studies in the mouse model evaluated local CMI. Again, extensive data emerged showing little, if any, role for local T cells in protection against infection (79, 185, 212). Local T cells are present (85, 100, 104, 154), albeit without any organized lymphoid tissue that is present at other mucosal tissues (i.e., Peyer's patches, etc.).

Immunoregulatory Mechanisms Play Key Roles

In light of the lack of any role for CMI against vaginitis, subsequent studies with mice aimed to identify the mechanism by which induced/present *Candida*-specific Th1-type cells were being prohibited from functioning at the vaginal mucosa. Data that emerged showed that a number of downregulatory mediators, including TGF-β, γ/δ T cells, Tregs, and plasmacytoid DCs, were present (212, 234). Finally, studies evaluating adhesion molecules on the vaginal endothelium and T cells in the draining lymph nodes showed that while the appropriate adhesion molecules were upregulated as expected on the surface on the vaginal endothelium following infection, the cells with the reciprocal homing receptors were decreased in the lymph nodes, possibly by circulating out or apoptosis (233). This served as an explanation for the lack of any T-cell infiltration into the vagina during infection. Thus, the overwhelming conclusion from the mouse studies is that CMI does not function against *C. albicans* at the vaginal mucosa due to significant immunoregulation. This concept is reasonable, since *Candida* as a commensal has evolved together with the host tissue to limit strong adaptive responses that could result in considerable inflammation at a reproductive site.

Role of Innate Immunity

Due to the lack of any demonstrable evidence for CMI or antibodies in defenses against *Candida* vaginitis, studies focused on the role of innate resistance. Several studies examined the role of PMNs, which were often erratically present in the vaginae of infected mice. Depletion of PMNs under estrogen or non-estrogen-treated conditions had no effect on vaginal fungal burden, and there were no correlations between numbers of PMNs in lavage fluids of infected mice and vaginal fungal burden (22, 79, 201). These results suggested that PMNs did not function similarly against *Candida* in the vaginal microenvironment compared to their function in blood or tissue culture. NK cells were also examined at the vaginal mucosa and found not to be present in naïve mice or following infection (167, 207). At the same time, the novel discovery that vaginal epithelial cells from mice, humans, and macaques had the ability to inhibit the growth of *C. albicans* was reported, potentially representing an innate host defense mechanism at the vaginal mucosa (16, 207, 208). This was paralleled by the identical activity of oral epithelial cells, as described above (205, 206). Studies to date show activity of vaginal epithelial cells similar in all respects to that of oral epithelial cells, with the exception that the anticandidal activity is lower (75 to 80% inhibition of growth for oral cells, versus 40 to 50% inhibition by vaginal cells) (161). In a study evaluating vaginal epithelial cell activity in women with RVVC and healthy women, the stage of the menstrual cycle did not influence the activity, but a significant reduction of vaginal epithelial cell antifungal activity was detected in those with RVVC at low effector-to-target ratios (16). Thus, there was also in vivo evidence to support vaginal epithelial cell antifungal activity as a protective mechanism. Interestingly, a study relative to *Candida*-vaginal epithelial cell interactions showed that mice deficient for alpha(1,2) fucosyltransferase ($Fut\text{-}2^{-/-}$) had higher fungal burdens than wild-type mice, suggesting that mucins with fucosylated glycans are involved in protection or at least adherence and subsequent responses by epithelial cells (61).

Identifying Critical Immune Factors of Susceptibility through Clinical Studies

Several clinical studies have centered on local immunity with the intention of identifying factors associated with susceptibility to infection. One study evaluated women with RVVC and healthy controls for Th cytokines and *Candida*-specific antibodies in vaginal secretions. Although Th cytokines in vaginal secretions of healthy women were generally of the Th1 type, a comprehensive evaluation of several Th and proinflammatory cytokines showed no unique pattern of cytokine deficiency for women with RVVC (78). In contrast, a recent study that evaluated only Th2-type cytokines and mediators showed increased IL-4, prostaglandin E_2, and *Candida*-specific IgE in lavage fluids from RVVC patients compared to asymptomatic women (224). While these results suggest some form of allergic reaction, which has always been postulated for a select group of women with RVVC, the disparity in results likely reflects a series of unavoidable problems with cross-sectional clinical studies. These include dynamics of the infection as women would attend the clinic at various times during the infection, lack of specificity of the cytokines measured for *C. albicans*, and potential influences of reproductive hormones. Hence, the clinical studies were at an impasse to explain any consistent immunodeficiency or to identify host immune factors associated with susceptibility or resistance to infection.

With these significant gaps remaining, a study evaluated adolescents for natural vaginal yeast colonization as well as for local and systemic immune sensitization. The findings revealed that adolescents were asymptomatically colonized with yeasts in the vagina at the high end of the normal range for adults (26%), had the same species distribution of *Candida* in the vagina (90% *C. albicans*), were fully immune sensitized to *Candida* systemically (peripheral blood responses and antibodies), and had detectable vaginal epithelial cell anti-*Candida* activity (14). Moreover, asymptomatically colonized adolescents demonstrated an extremely high vaginal fungal burden (50% had between 200 and 1,000 CFU, compared to 95% of adults having <200 CFU). Most interesting, however, was that the attack rate of VVC in this population (in a longitudinal study over 3 years) was <3%. Thus, the adolescents could maintain large numbers of *Candida* organisms vaginally without symptomatic infection, suggesting strong protective activity/responsiveness.

In light of all these findings, a safe but provocative live-challenge approach was designed to study the natural history of *C. albicans* vaginal infection. The results of the studies to date have reshaped much of what was understood relative to host defense against vaginitis (76). In a very conservative approach, women with no history of vaginitis were inoculated with small numbers of *Candida* organisms in a bolus. Very few women became symptomatic for vaginitis. Even broadening the inoculating conditions to include higher inocula given at different stages of the menstrual cycle and inclusion of growth-promoting conditions in the inoculum vehicle (growth media, glucose, and estrogen) had no effect. In contrast, inoculating women with documented infrequent episodes of VVC (caused by antibiotic usage, oral contraceptive usage, pregnancy, etc.) led to higher incidences of symptomatic infections. The vast majority of these symptomatic infections had a heavy PMN infiltrate that correlated with the observed symptoms of vaginitis (none of which were defined as severe). In fact, PMN infiltration showed a positive correlation with high vaginal fungal burden. Conversely, the women in these studies who presented as asymptomatic showed no evidence of PMN infiltration. In related studies, vaginal lavage fluid from women with symptomatic infection, but not those asymptomatically colonized or not colonized, stimulated the migration of PMNs in a chemotaxis assay (76). Furthermore, in studies evaluating vaginal epithelial cells for anti-*Candida* activity, women who became asymptomatically colonized or not colonized had significantly higher antifungal activity than those who became symptomatically infected (15).

Hence, the new hypothesis is that VVC is associated with signals following *Candida*-vaginal epithelial cell interactions that promote a nonprotective inflammatory leukocytic response and concomitant clinical symptoms, while resistance to VVC is associated with a lack of signals and/or noninflammatory antifungal activity by vaginal epithelial cells. Accordingly, a threshold number of organisms in the vagina is crucial to the epithelial cell-mediated signal(s) in the face of T-cell immunoregulation, and this threshold is different for different groups of women. In effect, the epithelial cells of women are either sensitive or insensitive to certain population numbers of *Candida* and secrete "danger" signals when the threshold of sensitivity is breached. This signal(s) stimulates PMN infiltration. For example, women with primary RVVC inevitably develop disease after completing a regimen of antifungal therapy. Vaginal epithelial cells in these women are extremely sensitive to *Candida* and signal the PMN infiltration after exposure to very low numbers of *Candida* organisms. Vaginal epithelial cells of women with an infrequent history of VVC have a lower sensitivity for *Candida* and thus do not signal PMNs until the population numbers of *Candida* grow, as, for example, following antibiotic therapy or hormone replacement therapy, following high-estrogen oral contraceptives, during pregnancy, or due to diabetes mellitus. Vaginal epithelial cells in women with no history of VVC are extremely insensitive to *Candida*. Thus, although the population numbers of *Candida* can increase with a reduced bacterial flora during antibiotic therapy or increased estrogen from oral contraceptives, they rarely, if ever, cross a threshold where the epithelial cells will stimulate PMN migration. Hence, symptoms of vaginitis are absent. This concept can be extended to adolescents, whose cellular threshold for *Candida* is expected to be high (similar to adults with no history of vaginitis). This would explain the high vaginal *Candida* numbers in adolescents with few symptomatic infections observed. A study designed to identify ranges of vaginal fungal burden in the different groups of women under conditions of a symptomatic infection with specific correlates to PMN infiltration will be required to formally test this hypothesis.

To this end, instead of susceptibility to infection being associated with a deficient or missing immune component and protection associated with a T-cell inflammatory response, it is now suggested that susceptibility to infection is associated with an acute inflammatory response, while resistance to infection is associated with a noninflammatory response. Thus, both resistance and susceptibility appear to be associated with innate immunity. And finally, the symptoms associated with vaginitis appear to be caused by the acute neutrophil response (host) rather than the organism. This may parallel data from neutropenic mice that had reduced inflammation during infection (22). It also renews the possible role for a genetic predisposition to infection. Indeed, studies evaluating gene polymorphisms in women with RVVC have shown a polymorphism in mannose-binding lectin-2 (MBL2) codon 54 allele B among white women

with a history of allergy (62, 89, 150). Another polymorphism in IL-4 has also been reported in RVVC that was associated with higher vaginal concentrations of IL-4 together with reduced nitric oxide and MBL (5).

Testing the New Paradigm in Animal Models

With the clinical data suggesting a significant paradigm shift for the pathogenesis of vaginal *Candida* infections, the established animal model was again employed to examine the PMN response closer and dissect the mechanisms involved. The characteristic erratic presence of PMNs in the lavage fluid of inoculated mice (79, 185) provided a foundation to work from. A careful analysis showed that on average, 60% of mice had high numbers of PMNs (>50/400× field), with the remainder having moderate/low/no PMNs. Although signs and symptoms of vaginitis are difficult to qualify or quantify in rodents, the presence of PMNs defined as a sign of infection/disease enabled the high-PMN animals to be considered those with a symptomatic condition and the low-PMN animals to be considered those with an asymptomatic condition. Lavage fluids collected from inoculated mice with high PMNs stimulated significant PMN migration compared to fluid from inoculated mice with low PMNs. A proteomic approach was used to identify the chemotactic factor(s) in lavage fluids with high PMNs (intense bands on polyacrylamide gel electrophoresis gels at 6 and 14 kDa) as calcium-binding proteins (CBPs) S100A8 and S100A9, also known as alarmins (241). These CBPs are known to be chemotactic for PMNs and are produced by a number of cell types, including epithelial cells and PMNs (88, 123, 184, 245). In addition, the heterodimer formed by S100A8 and S100A9 is termed calprotectin, a well-known antimicrobial peptide (215, 245). Both CBPs were identified further in lavage fluids by Western blotting and enzyme-linked immunosorbent assay (241). Production of the CBPs specifically by vaginal epithelial cells was confirmed by PCR. Finally, antibody neutralization employed in the chemotaxis assay showed that S100A8, but not S100A9, in lavage fluids from mice with high PMNs was responsible for PMN chemotaxis (241). While clinical studies in the area of CBPs and PMN chemotaxis during vaginitis are only in their infancy, proteomic analyses of lavage fluids from women with symptomatic infection have shown the presence of S100 CBPs, and as mentioned above, these lavage fluids stimulate the migration of PMNs in vitro (76). Thus, CBPs (alarmins) appear likely candidates triggered by sensitive epithelial cells to stimulate the migration of PMNs that initiate the inflammatory response. An amplified response may come from additional production of CBPs by the infiltrating PMNs. Current studies are focused on the identification of the PRRs responsible for epithelial cell triggering.

Most Recent Developments in Host Immunity

An interesting report was recently published describing a family of four women who had an early stop codon mutation in Tyr238X of dectin-1, a PRR that interacts with β-glucan on *Candida* to stimulate host defense. This mutation led to defective IL-17 production as well as TNF-α and IL-6 in response to *Candida* (73). Hence, these women were characterized as having a deficient Th17 response. Interestingly, the women were all affected by RVVC and onychomycosis, but not OPC or systemic candidiasis, suggesting that the Th17 response was protective against RVVC but not OPC. In related animal studies (described above), however, the Th17 cell lineage was protective against OPC (determined by enhanced susceptibility to infection in IL-23$^{-/-}$ and IL-17R$^{-/-}$ mice) (46). Related, vaginal inoculation of IL-23$^{-/-}$ mice showed equivalent vaginal fungal burden, PMN infiltration, and vaginal CBP S100A8/A9 production, suggesting a lack of a role for the Th17 lineage in host defense against vaginal candidiasis (Fidel and coworkers, unpublished data). Clearly, more studies need to be done to understand these data and determine a role, if any, for Th17 responses in protection or susceptibility to VVC or RVVC.

Conclusion: Using Data Collected To Devise Immunotherapeutic Strategies

Once the mechanism(s) for host susceptibility is fully understood, definitive new immunotherapeutic strategies can be developed that may reduce or prevent the incidence of VVC and RVVC. Current preclinical therapies under investigation include "protective antibodies" as a vaccine or treatment (93), vaccines using mannoprotein (MP) extract antigens, Saps, β-glucan, or the N terminus of Als3p adhesin (54, 170, 173, 202); anti-idiotypic antibodies with a mirror image of the yeast killer toxin (143); and gene therapy with adenovirus-encoding cytokines to overcome the immunoregulation of CMI (236). But if indeed the PMNs are the main instigator of the symptoms associated with vaginitis, immunotherapy to eliminate/block/neutralize the chemotactic signals associated with the PMN infiltration (i.e., S100 alarmin proteins) may be used to alleviate the symptoms associated with vaginitis, leaving the organism to exist as the harmless commensal it began as.

THE EMERGING ROLE OF MUCOSAL *CANDIDA* BIOFILMS AND HOST IMMUNITY

Recently there has been a tremendous interest in the role of biofilms in infectious diseases. It is estimated that 80% of human infections result from pathogenic biofilms (155). Biofilms are communities of microorganisms that are embedded in extracellular matrix (ECM), forming a complex microbial community. A unique feature of *C. albicans* biofilms is the morphological heterogeneity of the biofilm cells, which results in a complex three-dimensional biofilm architecture (42). Biofilm formation is dependent on the ability to undergo morphogenesis; mutants defective in hyphal formation in vitro are also defective in biofilm formation (160, 175). *C. albicans* biofilms have a unique gene expression pattern (243) and are more resistant to antifungal treatment than planktonic cells (121, 175). Therefore, in a clinical setting, biofilms represent a significant risk and are difficult to eradicate.

Candida biofilms have been studied primarily on abiotic surfaces (24, 174). A large proportion of *C. albicans* infections involve biofilms, which can form on a variety of synthetic polymers used in medical devices (60, 66, 121). Significant attention has been given to *Candida* biofilm formation of indwelling catheters, which can lead to life-threatening systemic infections (50, 60, 121). *Candida* spp. are the fourth leading cause of bloodstream infections and the third most commonly isolated organisms from intravascular catheters, and they are associated with the highest incidence of mortality (50, 230). *Candida* biofilm development on abiotic surfaces can be divided into several growth stages, including early, intermediate, and mature (42). During early biofilm formation, yeast cells adhere to an appropriate surface and initiate germ tube formation. The intermediate phase is characterized by continued hyphal elongation and ECM production, which consists of cell wall

polysaccharides and protein (6, 157). Mature biofilms consist of a yeast base, with hyphal elements encased in ECM extending away from the surface. In addition, persister cells are present, which are a multidrug-tolerant subpopulation of the biofilm (125). Newly formed daughter yeast cells grow out of hyphal elements and are released (dispersal), seeding new niches for biofilm formation or infection.

Candida biofilm formation on biotic surfaces has not been investigated until recently. *Candida* resides at mucosal surfaces as a normal inhabitant of the microbiota. Generally, mucosal disease is associated with a shift towards hyphal growth during vaginitis, oral candidiasis, and invasive GI tract infections (20, 33, 199). This provides some evidence that biofilm formation could occur on mucosal tissues and may be associated with disease. Alternatively, biofilm growth could represent a reservoir of chronic and/or persistent colonizing organisms that serve as a source of opportunistic infection. Several animal models of infection have recently been used or established to study *Candida* mucosal biofilm formation, including OPC (65), vaginitis (95), and DS (Fidel and coworkers, unpublished). Although it is too early to speculate on the requirement for biofilm formation in pathogenesis, these studies underscore the importance of the biofilm lifestyle during infection and provide a new level of complexity for pathogenesis and host response.

During OPC, *C. albicans* forms white pseudomembranous lesions on the oral mucosa, which consist of blastospores, pseudohyphae, and hyphae attached to underlying epithelium (1). It has been hypothesized that these plaques are biofilms, but this concept had not been studied in vivo. Using an immunosuppressed-mouse model, researchers were able to induce development of characteristic white lesions on the surface of the tongue after inoculation with *C. albicans* (65). Three-dimensional confocal imaging revealed a complex architecture similar to in vitro biofilms, with considerable β-glucan being exposed during invasion of the epithelium. This masking and unmasking of β-glucan in vivo are hypothesized to play a role in immunomodulation, because it is a dectin-1 ligand (226). Another interesting observation was the contribution of host components to the biofilm and ECM. Both keratin and PMN aggregates were observed within the ECM, suggesting that ECM may inhibit migration of effector cells.

Previous animal models of *Candida*-associated DS, dating back to 1978, used now-antiquated denture systems and did not explore the role of biofilm formation in disease (165). However, now with a potential role of biofilm formation on mucosal tissue contributing to pathogenesis of disease, and the well-known fact that *Candida* forms biofilms on denture materials, it seemed prudent to examine the role of biofilm formation in DS. Accordingly, a novel contemporary rat model of DS was developed using a custom fitted denture system composed of both fixed and magnetic removable plates (127). The denture system was installed against the hard palate of rats without alteration of the dental architecture. The novel design of this denture system (removable portion) allows for longitudinal studies to evaluate the progression of the disease. Biofilm formation was analyzed on the denture and palate via scanning electron and confocal microscopy (Johnson et al., unpublished). Biofilm formation on the denture occurred by week 4 postinoculation, characterized by the presence of yeast cells and hyphae coated with ECM. However, on the palate tissue, only blastospore colonization was observed, with no clinical evidence of disease. By week 6 postinoculation, biofilm formation was observed on both the denture and the palate tissue, and palatal erythema was evident. This suggests that during DS, *C. albicans* biofilm formation occurs initially on the denture plate, which in turn seeds the palatal tissue, resulting in mucosal biofilm formation and signs of disease.

Vaginal mucosal bacterial biofilms have been previously described for *Gardnerella vaginalis*, which causes vaginosis (193, 210, 216). However, in VVC and RVVC it was unknown whether *C. albicans* exists as a biofilm on the vaginal mucosa and, if so, whether the biofilm plays a role in immunopathogenesis. In vivo and ex vivo murine vaginitis models were recently used to examine mucosal biofilm formation by scanning electron and confocal microscopy. Wild-type *C. albicans* strains formed biofilms on the vaginal mucosa in vivo and ex vivo, as indicated by high fungal burden and microscopic analysis demonstrating typical biofilm architecture and ECM that colocalized with the presence of fungi. In contrast, mutants for a regulator of hyphal formation (*efg1/efg1*) and biofilm formation (*bcr1/bcr1*) exhibited weak to no biofilm formation and ECM production in both models despite comparable colonization levels. This raises interesting questions. Does the presence of a biofilm determine whether *C. albicans* behaves as a pathogen and allow the switch from commensalism? Does a biofilm influence the host response (i.e., CBP-dependent PMN migration to the vagina)? The fact that biofilm-deficient mutants still colonize vaginal mucosa will allow future studies to address the role of colonization versus biofilm formation during pathogenesis of vaginitis.

The development of animal models has been essential in demonstrating the ability of *Candida* to form biofilms on mucosal tissues in vivo. Advances in microscopic techniques will aid in the assessment of biofilm architecture in situ, as well as identify host and bacterial factors that coassociate within the biofilm. It will be especially important to monitor expression profiles of in vivo mucosal biofilms, which may differ significantly from in vivo abiotic biofilms or in vitro biofilms. It has been shown that *C. albicans* alternatively regulates its gene expression in the oral cavity compared with the GI tract (228). Therefore, virulence needs to be evaluated in terms of site-specific roles and in terms of colonization versus biofilm growth. It is likely that site-specific immunity plays a large role in determining how *C. albicans* responds within each microenvironment and tailors gene expression to maintain commensal status and/or biofilm growth. The role of the biofilm in immune evasion and manipulation of immune responses is of utmost importance for future studies and will likely direct novel therapies that target this immune and drug-resistant mode of growth.

CONCLUSION

If there remained any assumption that host defenses against *Candida* at one mucosal site are similar to those at another mucosal site, this review should finally put it to rest (Table 1). While it is recognized that evaluating site-specific immune responses will not always be easy, it remains critical to a fuller understanding of the true host response for each infection. Such concepts should serve as a paradigm shift of sorts or roadmap for how host defenses should be envisioned moving forward. This paradigm shift will also be important for vaccine research. Development of vaccines must consider the site-specific host responses and target the site of infection with the vaccine. It is already well known that immunization at systemic sites does not affect mucosal sites, whereas immunization of mucosal sites usually affects both

TABLE 1 Host defenses against mucosal *Candida* infections[a]

Type of immunity	Cell type or response	GI	Oral	Vaginal
Innate	PMNs	+/–	+/?	++[b]
	Macrophages	+/–	—	—
	NK cells	—	—	—
	Epithelial cells	?	++	++
	Inflammasome	?	++	?
	Th17 pathway	++	+++	—
Humoral	IgM	—/?	—	+/–?
	IgG	—/?	—	+/–?
	IgA	—/?	—/?	+/–?
Cell mediated	$CD4^+$ T cells (Th1)	++	++	—
	$CD8^+$ T cells[c]	+	++	—

[a]Symbols: +/–, data for or against; +/–?, data for or against, but questions remain; +, some role; ++, strong role; +++, very strong role; —, no role; ?, unknown.
[b]Activity is associated with susceptibility to infection rather than protection.
[c]May be innate response rather than adaptive.

mucosal and systemic sites. But the problem is still how best to stimulate strong responses at mucosal sites. Solving the problem remains a challenge for vaccine research targeting protection against *Candida* infections or other medically important fungi. Yet suffice it to say that studies that address host responses at the sites of infection, as well as the role of mucosal biofilms, will do much to advance our understanding of immunity against fungal infections, the pathogenesis of fungal infections, and the development of effective therapies to protect against them.

REFERENCES

1. **Allen, C. M., and F. M. Beck.** 1987. Differences in mucosal reaction related to *Candida albicans* isolates. *J. Oral Pathol.* **16:**89–93.
2. **Allen, C. M., A. Saffer, R. K. Meister, F. M. Beck, and S. Bradway.** 1994. Comparison of a lesion-inducing isolate and a non-lesional isolate of *Candida albicans* in an immunosuppressed rat model of oral candidiasis. *J. Oral Pathol. Med.* **23:**133–139.
3. **Anaissie, E. J., and G. P. Bodey.** 1990. Fungal infections in patients with cancer. *Pharmacotherapy* **10:**164S–169S.
4. **Aujla, S. J., P. J. Dubin, and J. K. Kolls.** 2007. Th17 cells and mucosal host defense. *Semin. Immunol.* **19:**377–382.
5. **Babula, O., G. Lazdane, J. Kroica, I. M. Linhares, W. J. Ledger, and S. S. Witkin.** 2005. Frequency of interleukin-4 (IL-4) -589 gene polymorphism and vaginal concentrations of IL-4, nitric oxide, and mannose-binding lectin in women with recurrent vulvovaginal candidiasis. *Clin. Infect. Dis.* **40:**1258–1262.
6. **Baillie, G. S., and L. J. Douglas.** 2000. Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J. Antimicrob. Chemother.* **46:**397–403.
7. **Balish, E., M. J. Balish, C. A. Salkowski, K. W. Lee, and K. F. Bartizal.** 1984. Colonization of congenitally athymic, gnotobiotic mice by *Candida albicans*. *Appl. Environ. Microbiol.* **47:**647–652.
8. **Balish, E., and H. Filutowicz.** 1991. Serum antibody response of gnotobiotic athymic and euthymic mice following alimentary tract colonization and infection with *Candida albicans*. *Can. J. Microbiol.* **37:**204–210.
9. **Balish, E., J. Jensen, T. Warner, J. Brekke, and B. Leonard.** 1993. Mucosal and disseminated candidiasis in gnotobiotic SCID mice. *J. Med. Vet. Mycol.* **31:**143–154.
10. **Balish, E., F. A. Vazquez-Torres, J. Jones-Carson, R. D. Wagner, and T. Warner.** 1996. Importance of β_2-microglobulin in murine resistance to mucosal and systemic candidiasis. *Infect. Immun.* **64:**5092–5097.
11. **Balish, E., R. D. Wagner, A. Vazquez-Torres, J. Jones-Carson, C. Pierson, and T. Warner.** 1999. Mucosal and systemic candidiasis in IL-8Rh-/- BALB/c mice. *J. Leukoc. Biol.* **66:**144–150.
12. **Balish, E., T. Warner, C. J. Pierson, D. M. Bock, and R. D. Wagner.** 2001. Oroesophageal candidiasis is lethal for transgenic mice with combined natural killer and T-cell defects. *Med. Mycol.* **39:**261–268.
13. **Balish, E., T. F. Warner, P. J. Nicholas, E. E. Paulling, C. Westwater, and D. A. Schofield.** 2005. Susceptibility of germfree phagocyte oxidase- and nitric oxide synthase 2-deficient mice, defective in the production of reactive metabolites of both oxygen and nitrogen, to mucosal and systemic candidiasis of endogenous origin. *Infect. Immun.* **73:**1313–1320.
14. **Barousse, M., B. J. Van Der Pol, D. Fortenberry, D. Orr, and P. L. Fidel, Jr.** 2004. Vaginal yeast colonization, prevalence of vaginitis, and associated local immunity in adolescents. *Sex. Transm. Infect.* **80:**48–53.
15. **Barousse, M. M., T. Espinosa, K. Dunlap, and P. L. Fidel, Jr.** 2005. Vaginal epithelial cell anti-*Candida albicans* activity is associated with protection against symptomatic vaginal candidiasis. *Infect. Immun.* **73:**7765–7767.
16. **Barousse, M. M., C. Steele, K. Dunlap, T. Espinosa, D. Boikov, J. D. Sobel, and P. L. Fidel, Jr.** 2001. Growth inhibition of *Candida albicans* by human vaginal epithelial cells. *J. Infect. Dis.* **184:**1489–1493.
17. **Bassetti, M., E. Righi, A. Costa, R. Fasce, M. P. Molinari, R. Rosso, F. B. Pallavicini, and C. Viscoli.** 2006. Epidemiological trends in nosocomial candidemia in intensive care. *BMC Infect. Dis.* **6:**21.
18. **Beno, D. W. A., and H. L. Mathews.** 1992. Growth inhibition of *Candida albicans* by interleukin-2-activated splenocytes. *Infect. Immun.* **60:**853–863.
19. **Beno, D. W. A., A. G. Stover, and H. L. Mathews.** 1995. Growth inhibition of *Candida albicans* hyphae by CD8+ lymphocytes. *J. Immunol.* **154:**5273–5281.

20. **Bilhan, H., T. Sulun, G. Erkose, H. Kurt, Z. Erturan, O. Kutay, and T. Bilgin.** 2009. The role of *Candida albicans* hyphae and *Lactobacillus* in denture-related stomatitis. *Clin. Oral Investig.* **13:**363–368.
21. **Black, C. A., F. M. Eyers, M. L. Dunkley, R. L. Clancy, and K. W. Beagley.** 1999. Major histocompatibility haplotype does not impact the course of experimentally induced murine vaginal candidiasis. *Lab. Anim. Sci.* **49:**668–672.
22. **Black, C. A., F. M. Eyers, A. Russell, M. L. Dunkley, R. L. Clancy, and K. W. Beagley.** 1998. Acute neutropenia decreases inflammation associated with murine vaginal candidiasis but has no effect on the course of infection. *Infect. Immun.* **66:**1273–1275.
23. **Black, C. A., F. M. Eyers, A. Russell, M. L. Dunkley, R. L. Clancy, and K. W. Beagley.** 1999. Increased severity of *Candida* vaginitis in BALB/c nu/nu mice versus the parent strain is not abrogated by adoptive transfer of T cell enriched lymphocytes. *J. Reprod. Immunol.* **45:**1–18.
24. **Blankenship, J. R., and A. P. Mitchell.** 2006. How to build a biofilm: a fungal perspective. *Curr. Opin. Microbiol.* **9:**588–594.
25. **Bonifazi, P., T. Zelante, C. D'Angelo, A. De Luca, S. Moretti, S. Bozza, K. Perruccio, R. G. Iannitti, G. Giovannini, C. Volpi, F. Fallarino, P. Puccetti, and L. Romani.** 2009. Balancing inflammation and tolerance in vivo through dendritic cells by the commensal *Candida albicans*. *Mucosal Immunol.* **2:**362–374.
26. **Budtz-Jorgensen, E., A. Stenderup, and M. Grabowski.** 1975. An epidemiologic study of yeasts in elderly denture wearers. *Commun. Dent. Oral* **3:**115–119.
27. **Burns, D. N., R. Tuomala, B. H. Chang, R. Hershow, H. Minkoff, E. Rodriquez, C. Zorrilla, H. Hammill, and J. Regan.** 1997. Vaginal colonization or infection with *Candida albicans* in human immunodeficiency virus-infected women during pregnancy and during postpartum period. *Clin. Infect. Dis.* **24:**201–210.
28. **Cain, J. A., and G. S. Deepe, Jr.** 2000. Interleukin-12 neutralization alters lung inflammation and leukocyte expression of CD80, CD86, and major histocompatibility complex class II in mice infected with *Histoplasma capsulatum*. *Infect. Immun.* **68:**2069–2076.
29. **Calderone, R. A., and W. A. Fonzi.** 2001. Virulence factors of *Candida albicans*. *Trends Microbiol.* **9:**327–335.
30. **Cameron, A. L., B. Kirby, W. Fei, and C. E. M. Griffiths.** 2002. Natural killer and natural killer-T cells in psoriasis. *Arch. Dermatol. Res.* **294:**363–369.
31. **Cantorna, M., D. Mook, and E. Balish.** 1990. Resistance of congenitally immunodeficient gnotobiotic mice to vaginal candidiasis. *Infect. Immun.* **58:**3813–3815.
32. **Cantorna, M. T., and C. Balish.** 1991. Acquired immunity to systemic candidiasis in immunodeficient mice. *J. Infect. Dis.* **164:**936–943.
33. **Cantorna, M. T., and E. Balish.** 1990. Mucosal and systemic candidiasis in congenitally immunodeficient mice. *Infect. Immun.* **58:**1093–1100.
34. Reference deleted.
35. **Casadevall, A.** 1995. Antibody immunity and invasive fungal infections. *Infect. Immun.* **63:**4211–4218.
36. **Cassone, A., M. Boccanera, D. A. Adriani, G. Santoni, and F. De Bernardis.** 1995. Rats clearing a vaginal infection by *Candida albicans* acquire specific, antibody-mediated resistance to vaginal infection. *Infect. Immun.* **63:**2619–2624.
37. **Cassone, A., F. De Bernardis, A. Torosantucci, E. Tacconelli, M. Tumbarello, and R. Cauda.** 1999. In vitro and in vivo anticandidal activity of human immunodeficiency virus protease inhibitors. *J. Infect. Dis.* **180:**448–453.
38. **Castro, G., and R. Martinez.** 2009. Relationship between serum and saliva antibodies to *Candida* and isolation of *Candida* species from the mucosa of HIV-infected individuals. *Mycoses* **52:**246–250.
39. **Cenci, E., A. Mencacci, G. Del Sero, A. Bacci, C. Montagnoli, C. F. D'Ostiani, P. Mosci, M. Bachmann, F. Bistoni, M. Kopf, and L. Romani.** 1999. Interleukin-4 causes susceptibility to invasive pulmonary aspergillosis through suppression of protective type I responses. *J. Infect. Dis.* **180:**1957–1968.
40. **Cenci, E., A. Mencacci, R. Spaccapelo, L. Tonnetti, P. Mosci, K. H. Enssle, P. Puccetti, L. Romani, and F. Bistoni.** 1995. T helper cell type 1 (Th1)- and Th2-like responses are present in mice with gastric candidiasis but protective immunity is associated with Th1 development. *J. Infect. Dis.* **171:**1279–1288.
41. **Chakir, J., L. Cote, C. Coulombe, and N. Deslauriers.** 1994. Differential pattern of infection and immune response during experimental oral candidiasis in BALB/c and DBA/2 (H-2d) mice. *Oral Microbiol. Immunol.* **9:**88–94.
42. **Chandra, J., D. M. Kuhn, P. K. Mukherjee, L. L. Hoyer, T. McCormick, and M. A. Ghannoum.** 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J. Bacteriol.* **183:**5385–5394.
43. Reference deleted.
44. **Clark, R. A., S. A. Blakley, J. Rice, and W. Brandon.** 1995. Predictors of HIV progression in women. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **9:**43–50.
45. **Concia, E., A. M. Azzini, and M. Conti.** 2009. Epidemiology, incidence and risk factors for invasive candidiasis in high-risk patients. *Drugs* **69**(Suppl. 1)**:**5–14.
46. **Conti, H. R., F. Shen, N. Nayyar, E. Stocum, J. N. Sun, M. J. Lindemann, A. W. Ho, J. H. Hai, J. J. Yu, J. W. Jung, S. G. Filler, P. Masso-Welch, M. Edgerton, and S. L. Gaffen.** 2009. Th17 cells and IL-17 receptor signaling are essential for mucosal host defense against oral candidiasis. *J. Exp. Med.* **206:**299–311.
47. **Coogan, M. M., S. P. Sweet, and S. J. Challacombe.** 1994. Immunoglobulin A (IgA), IgA1, and IgA2 antibodies to *Candida albicans* in whole and parotid saliva in human immunodeficiency virus infection and AIDS. *Infect. Immun.* **62:**892–896.
48. **Corrigan, E. M., R. L. Clancy, M. L. Dunkley, F. M. Eyers, and K. W. Beagley.** 1998. Cellular immunity in recurrent vulvovaginal candidiasis. *Clin. Exp. Immunol.* **111:**574–578.
49. **Croxtall, J. D., D. W. Gilroy, E. Solito, Q. Choudhury, B. J. Ward, J. C. Buckingham, and R. J. Flower.** 2003. Attenuation of glucocorticoid functions in an Anx-A1 -/- cell line. *Biochem. J.* **371:**927–935.
50. **Crump, J. A., and P. J. Collignon.** 2000. Intravascular catheter-associated infections. *Eur. J. Clin. Microbiol. Infect. Dis.* **19:**1–8.
51. **Daniluk, T., G. Tokajuk, W. Stokowska, K. Fiedoruk, M. Sciepuk, M. L. Zaremba, D. Rozkiewicz, D. Cylwik-Rokicka, B. A. Kedra, I. Anielska, M. Gorska, and B. R. Kedra.** 2006. Occurrence rate of oral *Candida albicans* in denture wearer patients. *Adv. Med. Sci.* **51**(Suppl. 1):77–80.
52. **Davis, S. L., J. A. Vazquez, and P. S. McKinnon.** 2007. Epidemiology, risk factors, and outcomes of *Candida albicans* versus non-*albicans* candidemia in nonneutropenic patients. *Ann. Pharmacother.* **41:**568–573.
53. **De Bernardis, F., M. Boccanera, D. Adriana, E. Spreghini, G. Santoni, and A. Cassone.** 1997. Protective role of antimannan and anti-aspartyl proteinase antibodies in an experimental model of *Candida albicans* vaginitis in rats. *Infect. Immun.* **65:**3399–3405.
54. **De Bernardis, F., M. Boccanera, D. Adriani, A. Girolamo, and A. Cassone.** 2002. Intravaginal and intranasal immunizations are equally effective in inducing vaginal antibodies and conferring protection against vaginal candidiasis. *Infect. Immun.* **70:**2725–2729.

55. **De Bernardis, F., G. Santoni, M. Boccanera, E. Spreghini, D. Adriani, L. Morelli, and A. Cassone.** 2000. Local anticandidal immune responses in a rat model of vaginal infection by and protection against *Candida albicans*. *Infect. Immun.* **68:**3297–3304.
56. **Del Sero, G., A. Mencacci, E. Cenci, C. F. d'Ostiani, C. Montagnoli, A. Bacci, P. Mosci, M. Kopf, and L. Romani.** 1999. Antifungal type 1 responses are upregulated in IL-10-deficient mice. *Microbes Infect.* **1:**1169–1180.
57. **De Repentigny, L., F. Aumont, J. Ripeau, M. Fiorillo, D. Kay, Z. Hanna, and P. Jolicoeur.** 2002. Mucosal candidiasis in transgenic mice expressing human immunodeficiency virus type 1. *J. Infect. Dis.* **185:**1103–1114.
58. **Deslauriers, N., L. Cote, S. Montplaisir, and L. De Repentigny.** 1997. Oral carriage of *Candida albicans* in murine AIDS. *Infect. Immun.* **65:**661–667.
59. **Diaz, L. S., H. Foster, M. R. Stone, S. Fujimura, D. A. Relman, and J. A. Levy.** 2005. VCAM-1 expression on CD8+ cells correlates with enhanced anti-HIV suppressing activity. *J. Immunol.* **174:**1574–1579.
60. **Dominic, R. M., S. Shenoy, and S. Baliga.** 2007. *Candida* biofilms in medical devices: evolving trends. *Kathmandu Univ. Med. J.* **5:**431–436.
61. **Domino, S. E., E. A. Hurd, K. A. Thomsson, D. M. Karnak, J. M. Holmén Larsson, E. Thomsson, M. Bäckström, and G. C. Hansson.** 2009. Cervical mucins carry alpha(1,2) fucosylated glycans that partly protect from experimental vaginal candidiasis. *Glycoconj. J.* **26:** 1125–1134.
62. **Donders, G. G., O. Babula, G. Bellen, I. M. Linhares, and S. S. Witkin.** 2008. Mannose-binding lectin gene polymorphism and resistance to therapy in women with recurrent vulvovaginal candidiasis. *BJOG* **115:**1225–1231.
63. **Dongari-Bagtzoglou, A., and H. Kashleva.** 2003. *Candida albicans* triggers interleukin-8 by oral epithelial cells. *Microb. Pathog.* **34:**169–177.
64. **Dongari-Bagtzoglou, A., and H. Kashleva.** 2003. Granulocyte-macrophage colony-stimulating factor responses of oral epithelial cells to *Candida albicans*. *Oral Microbiol. Immunol.* **18:**165–170.
65. **Dongari-Bagtzoglou, A., H. Kashleva, P. Dwivedi, P. Diaz, and J. Vasilakos.** 2009. Characterization of mucosal *Candida albicans* biofilms. *PloS One* **4:**e7967.
66. **Douglas, L. J.** 2003. *Candida* biofilms and their role in infection. *Trends Microbiol.* **11:**30–36.
67. **Duerr, A., M. F. Sierra, J. Feldman, L. M. Clarke, I. Ehlich, and J. Dehovitz.** 1997. Immune compromise and prevalence of *Candida* vulvovaginitis in human immunodeficiency virus-infected women. *Obstet. Gynecol.* **90:**252–256.
68. **Elahi, S., G. Pang, R. Clancy, and R. B. Ashman.** 2000. Cellular and cytokine correlates of mucosal protection in murine model of oral candidiasis. *Infect. Immun.* **68:**5771–5777.
69. **El Hed, A., A. Khaitan, L. Kozhaya, N. Manel, D. Daskalakis, W. Borkowsky, F. Valentine, D. R. Littman, and D. Unutmaz.** 2010. Susceptibility of human Th17 cells to human immunodeficiency virus and their perturbation during infection. *J. Infect. Dis.* **201:**843–854.
70. **Farah, C. S., S. Elahi, G. Pang, T. Gotjamanos, G. J. Seymour, R. L. Clancy, and R. B. Ashman.** 2001. T cells augment monocyte and neutrophil function in host resistance against oropharyngeal candidiasis. *Infect. Immun.* **69:**6110–6118.
71. **Farah, C. S., T. Gotjamanos, G. J. Seymour, and R. B. Ashman.** 2002. Cytokines in the oral mucosa of mice infected with *Candida albicans*. *Oral Microbiol. Immunol.* **17:**375–378.
72. **Feng, Z., B. Jiang, J. Chandra, M. Ghannoum, S. Nelson, and A. Weinberg.** 2005. Human beta-defensins: differential activity against candidal species and regulation by *Candida albicans*. *J. Dent. Res.* **84:**445–450.
73. **Ferwerda, B., G. Ferwerda, T. S. Plantinga, J. A. Willment, A. B. van Spriel, H. Venselaar, C. C. Elbers, M. D. Johnson, A. Cambi, C. Huysamen, L. Jacobs, T. Jansen, K. Verheijen, L. Masthoff, S. A. Morre, G. Vriend, D. L. Williams, J. R. Perfect, L. A. Joosten, C. Wijmenga, J. W. van der Meer, G. J. Adema, B. J. Kullberg, G. D. Brown, and M. G. Netea.** 2009. Human dectin-1 deficiency and mucocutaneous fungal infections. *N. Engl. J. Med.* **361:**1798–1801.
74. **Fidel, P. L.** 2006. *Candida*-host interactions in HIV disease: relationships in oropharyngeal candidiasis. *Adv. Dent. Res.* **19:**80–84.
75. **Fidel, P. L., Jr.** 2004. History and new insights into host defense against vaginal candidiasis. *Trends Microbiol.* **12:**220–227.
76. **Fidel, P. L., Jr., M. Barousse, T. Espinosa, C. Camaratti, M. Ficarra, D. H. Martin, A. J. Quayle, and K. Dunlap.** 2004. A live intravaginal *Candida* challenge in humans reveals new hypotheses for the immunopathogenesis of vulvovaginal candidiasis. *Infect. Immun.* **72:**2939–2946.
77. **Fidel, P. L., Jr., J. L. Cutright, and J. D. Sobel.** 1995. Effects of systemic cell-mediated immunity on vaginal candidiasis in mice resistant and susceptible to *Candida albicans* infections. *Infect. Immun.* **63:**4191–4194.
78. **Fidel, P. L., Jr., K. A. Ginsburg, J. L. Cutright, N. A. Wolf, D. Leaman, K. Dunlap, and J. D. Sobel.** 1997. Vaginal-associated immunity in women with recurrent vulvovaginal candidiasis: evidence for vaginal Th1-type responses following intravaginal challenge with *Candida* antigen. *J. Infect. Dis.* **176:**728–739.
79. **Fidel, P. L., Jr., W. Luo, C. Steele, J. Chabain, M. Baker, and F. L. Wormley.** 1999. Analysis of vaginal cell populations during experimental vaginal candidiasis. *Infect. Immun.* **67:**3135–3140.
80. **Fidel, P. L., Jr., M. E. Lynch, D. H. Conaway, L. Tait, and J. D. Sobel.** 1995. Mice immunized by primary vaginal *Candida albicans* infection develop acquired vaginal mucosal immunity. *Infect. Immun.* **63:**547–553.
81. **Fidel, P. L., Jr., M. E. Lynch, V. Redondo-Lopez, J. D. Sobel, and R. Robinson.** 1993. Systemic cell-mediated immune reactivity in women with recurrent vulvovaginal candidiasis (RVVC). *J. Infect. Dis.* **168:**1458–1465.
82. **Fidel, P. L., Jr., M. E. Lynch, and J. D. Sobel.** 1995. Circulating CD4 and CD8 T cells have little impact on host defense against experimental vaginal candidiasis. *Infect. Immun.* **63:**2403–2408.
83. **Fidel, P. L., Jr., M. E. Lynch, and J. D. Sobel.** 1994. Effects of preinduced *Candida*-specific systemic cell-mediated immunity on experimental vaginal candidiasis. *Infect. Immun.* **62:**1032–1038.
84. **Fidel, P. L., Jr., and J. D. Sobel.** 1996. Immunopathogenesis of recurrent vulvovaginal candidiasis. *Clin. Microbiol. Rev.* **9:**335–348.
85. **Fidel, P. L., Jr., N. A. Wolf, and M. A. KuKuruga.** 1996. T lymphocytes in the murine vaginal mucosa are phenotypically distinct from those in the periphery. *Infect. Immun.* **64:**3793–3799.
86. **Fong, I. W., P. McCleary, and S. Read.** 1992. Cellular immunity of patients with recurrent or refractory vulvovaginal moniliasis. *Am. J. Obstet. Gynecol.* **166:** 887–890.
87. **Frank, C. F., and M. K. Hostetter.** 2007. Cleavage of E-cadherin: a mechanism for disruption of the intestinal epithelial barrier by *Candida albicans*. *Transl. Res.* **149:**211–222.
88. **Gebhardt, C., J. Németh, P. Angel, and J. Hess.** 2006. S100A8 and S100A9 in inflammation and cancer. *Biochem. Pharmacol.* **72:**1622–1631.

89. **Giraldo, P. C., O. Babula, A. K. Goncalves, I. M. Linhares, R. L. Amaral, W. J. Ledger, and S. S. Witkin.** 2007. Mannose-binding lectin gene polymorphism, vulvovaginal candidiasis, and bacterial vaginosis. *Obstet. Gynecol.* **109:**1123–1128.
90. **Goupil, M., E. Belanger Trudelle, V. Dugas, C. Racicot-Bergeron, F. Aumont, S. Senechal, Z. Hanna, P. Jolicoeur, and L. De Repentigny.** 2009. Macrophage-mediated responses to *Candida albicans* in mice expressing the human immunodeficiency virus type 1 transgene. *Infect. Immun.* **77:**4136–4149.
91. **Greenspan, D., E. Komaroff, M. Redford, J. A. Phelan, M. Navazesh, M. E. Alves, H. Kamrath, R. Mulligan, C. E. Barr, and J. S. Greenspan.** 2000. Oral mucosal lesions and HIV viral load in the Women's Interagency HIV Study (WIHS). *J. Acquir. Immune Defic. Syndr.* **25:**44–50.
92. **Gruber, A., C. Speth, E. Lukasser-Vogl, R. Zangerle, M. Borg-von Zepelin, M. P. Dierich, and R. Wurzner.** 1999. Human immunodeficiency virus type 1 protease inhibitor attenuates *Candida albicans* virulence properties in vitro. *Immunopharmacology* **41:**227–234.
93. **Han, Y., R. P. Morrison, and J. E. Cutler.** 1998. A vaccine and monoclonal antibodies that enhance mouse resistance to *Candida albicans* vaginal infection. *Infect. Immun.* **66:**5771–5776.
94. **Han, Y., M. H. Riesselman, and J. E. Cutler.** 2000. Protection against candidiasis by an immunoglobulin G3 (IgG3) monoclonal antibody specific for the same mannotriose as an IgM protective antibody. *Infect. Immun.* **68:**1649–1654.
95. **Harriott, M. M., E. A. Lilly, T. E. Rodriguez, P. L. Fidel, Jr., and M. C. Noverr.** 2010. *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiology* **156**(Pt. 12)**:**3635–3644.
96. **Hector, R. F., J. E. Domer, and E. W. Carrow.** 1982. Immune responses to *Candida albicans* in genetically distinct mice. *Infect. Immun.* **38:**1020–1028.
97. **Hise, A. G., J. Tomalka, S. Ganesan, K. Patel, B. A. Hall, G. D. Brown, and K. A. Fitzgerald.** 2009. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. *Cell Host Microbe* **5:**487–497.
98. **Hladik, F., G. Lentz, E. Delpit, A. McElroy, and M. J. McElrath.** 1999. Coexpression of CCR5 and IL-2 in human genital but not blood cells: implications for the ontogeny of the CCR5+ Th1 phenotype. *J. Immunol.* **163:**2306–2313.
99. **Hobbs, J. R., D. Briden, F. Davidson, M. Kahan, and J. K. Oates.** 1977. Immunological aspects of candidal vaginitis. *Proc. R. Soc. Med.* **70:**11–14.
100. **Ibraghimov, A. R., R. E. Sacco, M. Sandor, Z. Iakoubov, and R. G. Lynch.** 1995. Resident CD4+ αβ T cells of the murine female genital tract: a phenotypically distinct T cell lineage that rapidly proliferates in response to systemic T cell activation stimuli. *Int. Immunol.* **7:**1763–1769.
101. **Iiai, T., H. Watanabe, T. Suda, H. Okamoto, T. Abo, and K. Hatakeyama.** 2002. CD161+ T (NT) cells exist predominately in human intestinal epithelium as well as liver. *Clin. Exp. Immunol.* **129:**92–98.
102. **Imam, N., C. C. J. Carpenter, K. H. Mayer, A. Fisher, M. Stein, and S. B. Danforth.** 1990. Hierarchical pattern of mucosal *Candida* infections in HIV-seropositive women. *Am. J. Med.* **89:**142–146.
103. **Inghirami, G., B. Y. Zhu, L. Chess, and D. M. Knowles.** 1990. Flow cytometric and immunohistochemical characterization of the gamma/delta T-lymphocyte population in normal human lymphoid tissue and peripheral blood. *Am. J. Pathol.* **136:**357–367.
104. **Itohara, S., A. G. Farr, J. J. Lafaille, M. Bonneville, Y. Takagaki, W. Haas, and S. Tonegawa.** 1990. Homing of a gamma/delta thymocyte subset with homogenous T-cell receptors to mucosal epithelia. *Nature* **343:**754–757.
105. **Jensen, J., A. Vazquez-Torres, and E. Balish.** 1992. Poly(I · C)-induced interferons enhance susceptibility of SCID mice to systemic candidiasis. *Infect. Immun.* **60:**4549–4557.
106. **Jensen, J., T. Warner, and E. Balish.** 1994. The role of phagocytic cells in resistance to disseminated candidiasis in granulocytopenic mice. *J. Infect. Dis.* **170:** 900–905.
107. **Jensen, J., T. Warner, and E. Balish.** 1993. Resistance of SCID mice to *Candida albicans* administered intravenously or colonizing the gut: role of polymorphonuclear leukocytes and macrophages. *J. Infect. Dis.* **167:**912–919.
108. **Jensen, J., T. Warner, C. Johnson, and E. Balish.** 1996. Oral immunization of mice against candidiasis. *J. Infect. Dis.* **174:**133–140.
109. **Johansson, E. L., A. Rudin, L. Wassen, and J. Holmgren.** 2000. Distribution of lymphocytes and adhesion molecules in human cervix and vagina. *Immunology* **96:**272–277.
110. Reference deleted.
111. **Jones-Carson, J., A. Vazquez-Torres, T. Warner, and E. Balish.** 2000. Disparate requirement for T cells in resistance to mucosal and acute systemic candidiasis. *Infect. Immun.* **68:**2363–2365.
112. **Kalo-Klein, A., and S. S. Witkin.** 1989. *Candida albicans*: cellular immune system interactions during different stages of the menstrual cycle. *Am. J. Obstet. Gynecol.* **161:**1132–1136.
113. **Kent, H. L.** 1991. Epidemiology of vaginitis. *Am. J. Obstet. Gynecol.* **165:**1168–1175.
114. **Kirkpatrick, C. H.** 1984. Host factors in defense against fungal infections. *Am. J. Med.* **77:**1–12.
115. **Kirkpatrick, C. H., J. W. Chandler, and R. N. Schimke.** 1970. Chronic mucocutaneous moniliasis with impaired delayed hypersensitivity. *Clin. Exp. Immunol.* **6:**375–385.
116. **Kirkpatrick, C. H., R. R. Rich, and J. E. Bennett.** 1971. Chronic mucocutaneous candidiasis: model building in cellular immunity. *Ann. Intern. Med.* **74:**955–978.
117. **Kirkpatrick, C. H., and P. G. Sohnle.** 1981. Chronic mucocutaneous candidiasis, p. 495–514. *In* B. Safai and R. A. Good (ed.), *Immunodermatology*. Plenum Press, New York, NY.
118. **Klein, R. S., C. A. Harris, C. B. Small, B. Moll, M. Lesser, and G. H. Friedland.** 1984. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **311:**354–357.
119. **Kleinegger, C. L., S. R. Lockhart, K. Vargas, and D. R. Soll.** 1996. Frequency, intensity, species, and strains of oral *Candida* vary as a function of host age. *J. Clin. Microbiol.* **34:**2246–2254.
120. **Koh, A. Y., J. R. Kohler, K. T. Coggshall, N. Van Rooijen, and G. B. Pier.** 2008. Mucosal damage and neutropenia are required for *Candida albicans* dissemination. *PLoS Pathog.* **4:**e35.
121. **Kojic, E. M., and R. O. Darouiche.** 2004. *Candida* infections of medical devices. *Clin. Microbiol. Rev.* **17:**255–267.
122. **Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo.** 2009. IL-17 and Th17 cells. *Annu. Rev. Immunol.* **27:**485–517.
123. **Kumar, R. K., Z. Yang, S. Bilson, S. Thliveris, B. E. Cooke, and C. L. Geczy.** 2001. Dimeric S100A8 in human neutrophils is diminished after phagocytosis. *J. Leukoc. Biol.* **70:**59–64.

124. **Kunkl, A., L. Mortara, M. T. Valle, D. Fenoglio, M. Paola, A. M. Megiovanni, A. Alessandrini, G. Li Pira, G. Mazzarello, V. Del Bono, A. Canessa, D. Bassetti, and F. Manca.** 1998. Recognition of antigenic clusters of *Candida albicans* by T lymphocytes from human immunodeficiency virus-infected persons. *J. Infect. Dis.* **178:**488–496.
125. **LaFleur, M. D., C. A. Kumamoto, and K. Lewis.** 2006. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob. Agents Chemother.* **50:**3839–3846.
126. **LeBlanc, D. M., M. M. Barousse, and P. L. Fidel, Jr.** 2006. A role for dendritic cells in immunoregulation during experimental vaginal candidiasis. *Infect. Immun.* **74:**3213–3221.
127. **Lee, H., A. Yu, C. C. Johnson, E. A. Lilly, M. C. Noverr, and P. L. Fidel, Jr.** 17 February 2011. Fabrication of a multi-applicable removable intraoral denture system for rodent research. *J. Oral Rehabil.* doi:10.1111/j.1365-2842.2011.02206.x.
128. **Lehrnbecher, T., C. Frank, K. Engels, S. Kriener, A. H. Groll, and D. Schwabe.** 2010. Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. *J. Infect.* **61**:259–265.
129. **Leigh, J. E., M. Barousse, R. K. Swoboda, T. Myers, S. Hager, N. A. Wolf, J. L. Cutright, J. Thompson, J. D. Sobel, and P. L. Fidel, Jr.** 2001. *Candida*-specific systemic cell-mediated immune reactivities in HIV-infected persons with and without mucosal candidiaisis. *J. Infect. Dis.* **183:**277–285.
130. **Leigh, J. E., K. M. McNulty, and P. L. Fidel, Jr.** 2006. Characterization of the immune status of $CD8^+$ T cells in oral lesions of human immunodeficiency virus-infected persons with oropharyngeal candidiasis. *Clin. Vaccine Immunol.* **13:**678–683.
131. **Leigh, J. E., C. Steele, F. L. Wormley, Jr., and P. L. Fidel, Jr.** 2001. Salivary cytokine profiles in the immunocompetent individual with *Candida*-associated denture stomatitis. *Oral Microbiol. Immunol.* **17:**311–314.
132. **Leigh, J. E., C. Steele, F. L. Wormley, Jr., W. Luo, R. A. Clark, W. R. Gallaher, and P. L. Fidel, Jr.** 1998. Th1/Th2 cytokine expression in saliva of HIV-positive and HIV-negative individuals: a pilot study in HIV-positive individuals with oropharyngeal candidiasis. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* **19:**373–380.
133. **Lilly, E., D. J. Hart, J. E. Leigh, S. Hager, K. M. McNulty, D. E. Mercante, and P. L. Fidel, Jr.** 2004. Tissue-associated cytokine expression in HIV-positive persons with oropharyngeal candidiasis. *J. Infect. Dis.* **190:**605–612.
134. **Lilly, E. A., J. E. Leigh, K. M. McNulty, S. H. Joseph, D. E. Mercante, and P. L. Fidel, Jr.** 2006. Chemokine receptor expression in HIV-positive persons with oropharyngeal candidiasis. *Oral Dis.* **12:**493–499.
135. **Lilly, E. A., J. Yano, and P. L. Fidel, Jr.** 2010. Annexin-A1 identified as the oral epithelial cell anti-*Candida* effector moiety. *Mol. Oral Microbiol.* **25:**293–304.
136. **Liu, N., Y. P. Zhang, S. Han, J. Pei, L. Y. Xu, P. Lu, C. B. Shields, and X. Xu.** 2007. Annexin A1 reduces inflammatory reaction and tissue damage through inhibition of phospholipase A 2 activation in adult rats following spinal cord injury. *J. Neuropathol. Exp. Neurol.* **66:**932–943.
137. **Lynch, M. E., J. D. Sobel, and P. L. Fidel, Jr.** 1996. Role of antifingal drug resistance in the pathogenesis of recurrent vulvovaginal candidiasis. *J. Med. Vet. Mycol.* **34:**337–339.
138. **Macher, A. M.** 1988. The pathology of AIDS. *Public Health Rep.* **103:**246–254.
139. **Mackewicz, C. E., B. K. Patterson, S. A. Lee, and J. A. Levy.** 2000. $CD8^+$ cell noncytotoxic anti-human immunodeficiency virus response inhibits expression of viral RNA but not reverse transcription or provirus integration. *J. Gen. Virol.* **81**(Pt. 5)**:**1261–1264.
140. **Magee, D. M., and R. A. Cox.** 1995. Roles of gamma interferon and interleukin-4 in genetically determined resistance to *Coccidioides immitis*. *Infect. Immun.* **63:**3514–3519.
141. **Magill, S. S., S. M. Swoboda, E. A. Johnson, W. G. Merz, R. K. Pelz, P. A. Lipsett, and C. W. Hendrix.** 2006. The association between anatomic site of *Candida* colonization, invasive candidiasis, and mortality in critically ill surgical patients. *Diagn. Microbiol. Infect. Dis.* **55:**293–301.
142. **Magill, S. S., S. M. Swoboda, C. E. Shields, E. A. Colantuoni, A. W. Fothergill, W. G. Merz, P. A. Lipsett, and C. W. Hendrix.** 2009. The epidemiology of *Candida* colonization and invasive candidiasis in a surgical intensive care unit where fluconazole prophylaxis is utilized: follow-up to a randomized clinical trial. *Ann. Surg.* **249:**657–665.
143. **Magliani, W., S. Conti, F. DeBernardis, M. Gerloni, D. Bertolotti, P. Mozzoni, A. Cassone, and L. Polonelli.** 1997. Therapeutic potential of antiidiotypic single chain antibodies with yeast killer toxin activity. *Nat. Biotechnol.* **15:**155–158.
144. **Manzoni, P., M. Mostert, M. L. Leonessa, C. Priolo, D. Farina, C. Monetti, M. A. Latino, and G. Gomirato.** 2006. Oral supplementation with *Lactobacillus casei* subspecies *rhamnosus* prevents enteric colonization by *Candida* species in preterm neonates: a randomized study. *Clin. Infect. Dis.* **42:**1735–1742.
145. **Marquis, M., D. Lewandowski, V. Dugas, F. Aumont, S. Senechal, P. Jolicoeur, Z. Hanna, and L. De Repentigny.** 2006. $CD8^+$ T cells but not polymorphonuclear leukocytes are required to limit chronic oral carriage of *Candida albicans* in transgenic mice expressing human immunodeficiency virus type 1. *Infect. Immun.* **74:**2382–2391.
146. **Mathur, S., G. Virella, J. Koistinen, E. O. Horger, T. A. Mahvi, and H. H. Fudenberg.** 1977. Humoral immunity in vaginal candidiasis. *Infect. Immun.* **15:**287–294.
147. **McNulty, K. M., J. Plianrungsi, J. E. Leigh, D. E. Mercante, and P. L. Fidel, Jr.** 2005. Characterization of the $CD8^+$ T cells and microenvironment in oral lesions of human immunodeficiency virus-infected persons with oropharyngeal candidiasis. *Infect. Immun.* **73:**3659–3667.
148. **Meiller, T. F., B. Hube, L. Schild, M. E. Shirtliff, M. A. Scheper, R. Winkler, A. Ton, and M. A. Jabra-Rizk.** 2009. A novel immune evasion strategy of *Candida albicans*: proteolytic cleavage of a salivary antimicrobial peptide. *PLoS One* **4:**e5309.
149. **Mendling, W., and U. Koldovsky.** 1996. Investigations by cell-mediated immunologic tests and therapeutic trials with thymopentin in vaginal mycoses. *Infect. Dis. Obstet. Gynecol.* **4:**225–231.
150. **Milanese, M., L. Segat, F. De Seta, D. Pirulli, A. Fabris, M. Morgutti, and S. Crovella.** 2008. MBL2 genetic screening in patients with recurrent vaginal infections. *Am. J. Reprod. Immunol.* **59:**146–151.
151. **Millon, L., C. Drobacheff, R. Piarroux, M. Monod, G. Reboux, R. Laurent, and D. Meillet.** 2001. Longitudinal study of anti-*Candida albicans* mucosal immunity against aspartic proteinases in HIV-infected patients. *J. Acquir. Immune Defic. Syndr.* **26:**137–144.
152. **Montagnoli, C., A. Bacci, S. Bozza, R. Gaziano, P. Mosci, A. H. Sharpe, and L. Romani.** 2002. B7/CD28-

dependent CD4+CD25+ regulatory T cells are essential components of the memory-protective immunity to *Candida albicans*. *J. Immunol.* **169:**6298–6308.
153. **Myers, T. A., J. E. Leigh, A. Arribas, S. Hager, R. A. Clark, E. Lilly, and P. L. Fidel, Jr.** 2003. Immunohistochemical evaluation of T cells in oral lesions from human immunodeficiency virus-positive persons with oropharyngeal candidiasis. *Infect. Immun.* **71:**956–963.
154. **Nandi, D., and J. P. Allison.** 1991. Phenotypic analysis and gamma/delta-T cell receptor repertoire of murine T cells associated with the vaginal epithelium. *J. Immunol.* **147:**1773–1778.
155. **National Institutes of Health.** 1999. *SBIR/STTR Study and Control of Microbial Biofilms*. PA-99-084. National Institutes of Health, Bethesda, MD.
156. **Nawrot, U., K. Grzybek-Hryncewicz, U. Zielska, A. Czarny, and J. Podwinska.** 2000. The study of cell-mediated immune response in recurrent vulvovaginal candidiasis. *FEMS Immunol. Med. Microbiol.* **29:**89–94.
157. **Nett, J., L. Lincoln, K. Marchillo, R. Massey, K. Holoyda, B. Hoff, M. VanHandel, and D. Andes.** 2007. Putative role of β-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob. Agents Chemother.* **51:**510–520.
158. **Newman, S. L., and A. Holly.** 2001. *Candida albicans* is phagocytosed, killed, and processed for antigen presentation by human dendritic cells. *Infect. Immun.* **69:** 6813–6822.
159. **Nielsen, H., K. D. Bentsen, L. Hojtved, E. H. Willemoes, F. Scheutz, M. Schiodt, K. Stoltze, and J. J. Pindborg.** 1994. Oral candidiasis and immune status of HIV-infected patients. *J. Oral Pathol. Med.* 23:140–143.
160. **Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell.** 2006. Critical role of Bcr1-dependent adhesins in C. *albicans* biofilm formation in vitro and in vivo. *PLoS Pathog.* **2:**e63.
161. **Nomanbhoy, F., C. Steele, J. Yano, and P. L. Fidel, Jr.** 2002. Vaginal and oral epithelial cell anti-*Candida* activity. *Infect. Immun.* **70:**7081–7088.
162. **Nucci, M., and E. Anaissie.** 2001. Revisiting the source of candidemia: skin or gut? *Clin. Infect. Dis.* **33:** 1959–1967.
163. **Odds, F. C.** 1988. Candida *and Candidosis*, p. 104–110. University Park Press, Baltimore, MD.
164. **Oliviera, M. A., L. P. Carvalho, S. Gomes Mde, O. Bacellar, T. F. Barros, and E. M. Carvalho.** 2007. Microbiological and immunological features of oral candidiasis. *Microbiol. Immunol.* **51:**713–719.
165. **Olsen, I., and O. Bondevik.** 1978. Experimental *Candida*-induced denture stomatitis in the Wistar rat. *Scand. J. Dent. Res.* **86:**392–398.
166. **Palella, F. J., Jr., K. M. Delaney, A. C. Moorman, M. O. Loveless, J. Fuhrer, G. A. Satten, D. J. Aschman, and S. D. Holmberg, and the HIV Outpatient Study Investigators.** 1998. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. *N. Engl. J. Med.* **338:**853–860.
167. **Parr, M. B., and E. L. Parr.** 1991. Langerhans cells and T lymphocyte subsets in the murine vagina and cervix. *Biol. Reprod.* **44:**491–498.
168. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
169. **Phelan, J. A., B. R. Salzman, G. H. Friendland, and R. S. Klein.** 1987. Oral findings in patients with acquired immune deficiency syndrome. *Oral Surg.* **64:**50–56.
170. **Pietrella, D., A. Rachini, A. Torosantucci, P. Chiani, A. J. Brown, F. Bistoni, P. Costantino, P. Mosci, C. d'Enfert, R. Rappuoli, A. Cassone, and A. Vecchiarelli.** A beta-glucan-conjugate vaccine and anti-beta-glucan antibodies are effective against murine vaginal candidiasis as assessed by a novel in vivo imaging technique. *Vaccine* **28:**1717–1725.
171. **Quinti, I., C. Palma, E. C. Guerra, M. J. Gomez, I. Mezzaroma, F. Aiuti, and A. Cassone.** 1991. Proliferative and cytotoxic responses to mannoproteins of *Candida albicans* by peripheral blood lymphocyes of HIV-infected subjects. *Clin. Exp. Immunol.* **85:**1–8.
172. **Rabeneck, L., M. M. Crane, J. M. Risser, C. E. Lacke, and N. P. Wray.** 1993. A simple clinical staging system that predicts progression to AIDS using CD4 count, oral thrush, and night sweats. *J. Gen. Int. Med.* **8:**5–9.
173. **Rahman, D., M. Mistry, S. Thavaraj, S. J. Challacombe, and J. R. Naglik.** 2007. Murine model of concurrent oral and vaginal *Candida albicans* colonization to study epithelial host-pathogen interactions. *Microbes Infect.* **9:** 615–622.
174. **Ramage, G., J. P. Martinez, and J. L. Lopez-Ribot.** 2006. *Candida* biofilms on implanted biomaterials: a clinically significant problem. *FEMS Yeast Res.* **6:**979–986.
175. **Ramage, G., K. VandeWalle, J. L. Lopez-Ribot, and B. L. Wickes.** 2002. The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol. Lett.* **214:**95–100.
176. **Rangel-Frausto, M. S., T. Wiblin, H. M. Blumberg, L. Saiman, J. Patterson, M. Rinaldi, M. Pfaller, J. E. Edwards, Jr., W. Jarvis, J. Dawson, and R. P. Wenzel.** 1999. National epidemiology of mycoses survey (NEMIS): variations in rates of bloodstream infections due to *Candida* species in seven surgical intensive care units and six neonatal intensive care units. *Clin. Infect. Dis.* **29:** 253–258.
177. **Rescigno, M., G. Rotta, B. Valzasina, and P. Ricciardi-Castagnoli.** 2001. Dendritic cells shuttle microbes across gut epithelial monolayers. *Immunobiology* **204:**572–581.
178. **Rescigno, M., M. Urbano, B. Valzasina, M. Francolini, G. Rotta, R. Bonasio, F. Granucci, J. P. Kraehenbuhl, and P. Ricciardi-Castagnoli.** 2001. Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat. Immunol.* **2:**361–367.
179. **Rhoads, J. L., D. C. Wright, R. R. Redfield, and D. S. Burke.** 1987. Chronic vaginal candidiasis in women with human immunodeficiency virus infection. *JAMA* **257:** 3105–3107.
180. **Romagnoli, P., N. Pimpinelli, M. Mori, P. A. Reichart, L. R. Eversole, and G. Ficarra.** 1997. Immunocompetent cells in oral candidiasis of HIV-infected patients: an immunohistochemical and electron microscopical study. *Oral Dis.* **3:**99–105.
181. **Romani, L.** 2002. Immunology of invasive candidiasis, p. 223–241. *In* R. A. Calderone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
182. **Romano, L.** 1996. Biological role of Th cell subsets in candidiasis, p. 114–137. *In* L. Adorini, K.-I. Arai, C. Berek, J. D. Capra, A. M. Schmitt-Verhulst, and B. H. Waksman (ed.), *Th1 and Th2 Cells in Health and Disease*. Karger, Farmington, CT.
183. **Rouabhia, M., G. Ross, N. Page, and J. Chakir.** 2002. Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to *Candida albicans* or lipopolysaccharide stimulation. *Infect. Immun.* **70:**7073–7080.
184. **Ryckman, C., K. Vandal, P. Rouleau, M. Talbot, and P. A. Tessier.** 2003. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J. Immunol.* **170:** 3233–3242.

185. **Saavedra, M., B. Taylor, N. W. Lukacs, and P. L. Fidel, Jr.** 1999. Local production of chemokines during experimental vaginal candidiasis. *Infect. Immun.* **67:**5820–5829.
186. **Samonis, G., A. Gikas, P. Toloudis, S. Maraki, G. Vrentzos, Y. Tselentis, N. Tsaparas, and G. Bodey.** 1994. Prospective study of the impact of broad-spectrum antibiotics on the yeast flora of the human gut. *Eur. J. Clin. Microbiol. Infect. Dis.* **13:**665–667.
187. **Sangeorzan, J. A., S. F. Bradley, X. He, L. T. Zarins, G. L. Ridenour, R. N. Tiballi, and C. A. Kauffman.** 1994. Epidemiology of oral candidiasis in HIV infected patients: colonization, infection, treatment, and emergence of fluconazole resistance. *Am. J. Med.* **97:**339–346.
188. **Santoni, G., M. Boccanera, D. Adriani, R. Lucciarini, C. Amantini, S. Morrone, A. Cassone, and F. De Bernardis.** 2002. Immune cell-mediated protection against vaginal candidiasis: evidence for a major role of vaginal $CD4^+$ T cells and possible participation of other local lymphocyte effectors. *Infect. Immun.* **70:**4791–4797.
189. **Schaller, M., R. Mailhammer, G. Grassl, C. A. Sander, B. Hube, and H. C. Korting.** 2002. Infection of human oral epithelia with *Candida* species induces cytokine expression correlated to the degree of virulence. *J. Investig. Dermatol.* **118:**652–657.
190. **Schmid, J., F. C. Odds, M. J. Wiselka, K. G. Nicholson, and D. R. Soll.** 1992. Genetic similarity and maintenance of *Candida albicans* strains from a group of AIDS patients, demonstrated by DNA fingerprinting. *J. Clin. Microbiol.* **30:**935–941.
191. **Schuman, P., S. Ohmit, J. D. Sobel, K. H. Mayer, V. Greene, A. Rompalo, and R. S. Klein.** 1998. Oral lesions among women living with or at risk for HIV infection. *Am. J. Med.* **104:**559–563.
192. **Schuman, P., J. D. Sobel, S. Ohmit, K. H. Mayer, A. Rompalo, A. Duerr, D. K. Smith, D. Warren, and R. S. Klein.** 1998. Mucosal candidal colonization and candidiasis in women with or at risk for human immunodeficiency virus infection. *Clin. Infect. Dis.* **27:**1161–1167.
193. **Scott, T. G., B. Curran, and C. J. Smyth.** 1989. Electron microscopy of adhesive interactions between *Gardnerella vaginalis* and vaginal epithelial cells, McCoy cells and human red blood cells. *J. Gen. Microbiol.* **135:**475–480.
194. **Shakir, B. S., M. V. Martin, and C. J. Smith.** 1981. Induced palatal candidosis in the Wistar rat. *Arch. Oral Biol.* **26:**787–793.
195. **Singhi, S., D. S. Rao, and A. Chakrabarti.** 2008. *Candida* colonization and candidemia in a pediatric intensive care unit. *Pediatr. Crit. Care Med.* **9:**91–95.
196. **Sobel, J. D.** 1988. Pathogenesis and epidemiology of vulvovaginal candidiasis. *Ann. N. Y. Acad. Sci.* **544:** 547–557.
197. **Sobel, J. D.** 1992. Pathogenesis and treatment of recurrent vulvovaginal candidiasis. *Clin. Infect. Dis.* **14:**S148–S153.
198. **Sobel, J. D., S. Faro, R. Force, B. Foxman, W. J. Ledger, P. R. Nyirjesy, B. D. Reed, and P. R. Summers.** 1998. Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. *Am. J. Obstet. Gynecol.* **178:**203–211.
199. **Sobel, J. D., G. Muller, and H. R. Buckley.** 1984. Critical role of germ tube formation in the pathogenesis of candidal vaginitis. *Infect. Immun.* **44:**576–580.
200. **Soll, D. R., R. Galask, J. Schmid, C. Hanna, K. Mac, and B. Morrow.** 1991. Genetic dissimilarity of commensal strains of *Candida* spp. carried in different anatomical locations of the same healthy women. *J. Clin. Microbiol.* **29:**1702–1710.
201. **Sonoda, Y., N. Mukaida, J. Wang, M. Shimada-Hiratsuka, M. Naito, T. Kasahara, A. Harada, M. Inoue, and K. Matsushima.** 1998. Physiologic regulation of postovulatory neutrophil migration into vagina in mice by a C-X-C chemokine. *J. Immunol.* **160:**6159–6165.
202. **Spellberg, B., A. S. Ibrahim, L. Lin, V. Avanesian, Y. Fu, P. Lipke, H. Otoo, T. Ho, and J. E. Edwards, Jr.** 2008. Antibody titer threshold predicts anti-candidal vaccine efficacy even though the mechanism of protection is induction of cell-mediated immunity. *J. Infect. Dis.* **197:**967–971.
203. **Spinillo, A., G. Michelone, C. Cavanna, L. Colonna, E. Capuzzo, and S. Nicola.** 1994. Clinical and microbiological characteristics of symptomatic vulvovaginal candidiasis in HIV-seropositive women. *Genitourin. Med.* **70:**268–272.
204. **Steele, C., and P. L. Fidel, Jr.** 2002. Cytokine and chemokine production by human oral and vaginal epithelial cells in response to *Candida albicans*. *Infect. Immun.* **70:**577–583.
205. **Steele, C., J. E. Leigh, R. K. Swoboda, and P. L. Fidel, Jr.** 2000. Growth inhibition of *Candida* by human oral epithelial cells. *J. Infect. Dis.* **182:**1479–1485.
206. **Steele, C., J. E. Leigh, R. K. Swoboda, H. Ozenci, and P. L. Fidel, Jr.** 2001. Potential role for a carbohydrate moiety in anti-*Candida* activity of human oral epithelial cells. *Infect. Immun.* **69:**7091–7099.
207. **Steele, C., H. Ozenci, W. Luo, M. Scott, and P. L. Fidel, Jr.** 1999. Growth inhibition of *Candida albicans* by vaginal cells from naive mice. *Med. Mycol.* **37:**251–260.
208. **Steele, C., M. Ratterree, and P. L. Fidel, Jr.** 1999. Differential susceptibility to experimental vaginal candidiasis in macaques. *J. Infect. Dis.* **180:**802–810.
209. **Stranford, S. A., J. Skurnick, D. Louria, D. Osmond, S. Y. Chang, J. Sninsky, G. Ferrari, K. Weinhold, C. Lindquist, and J. A. Levy.** 1999. Lack of infection in HIV-exposed individuals is associated with a strong $CD8^+$ cell noncytotoxic anti-HIV response. *Proc. Natl. Acad. Sci. USA* **96:**1030–1035.
210. **Swidsinski, A., W. Mendling, V. Loening-Baucke, S. Swidsinski, Y. Dörffel, J. Scholze, H. Lochs, and H. Verstraelen.** 2008. An adherent *Gardnerella vaginalis* biofilm persists on the vaginal epithelium after standard therapy with oral metronidazole. *Am. J. Obstet. Gynecol.* **198:**97.e91–97.e96.
211. **Tachibana, T., T. Matsuyama, and M. Mitsuyama.** 1999. Involvement of CD4+ T cells and macrophages in acquired protection against infection with *Sporothrix schenckii* in mice. *Med. Mycol.* **37:**397–404.
212. **Taylor, B. N., M. Saavedra, and P. L. Fidel, Jr.** 2000. Local Th1/Th2 cytokine production during experimental vaginal candidiasis. *Med. Mycol.* **38:**419–431.
213. **Thompson, G. R., III, P. K. Patel, W. R. Kirkpatrick, S. D. Westbrook, D. Berg, J. Erlandsen, S. W. Redding, and T. F. Patterson.** 2010. Oropharyngeal candidiasis in the era of antiretroviral therapy. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **109:**488–495.
214. **Torres, S. R., A. Garzino-Demo, T. F. Meiller, V. Meeks, and M. A. Jabra-Rizk.** 2009. Salivary histatin-5 and oral fungal colonisation in HIV+ individuals. *Mycoses* **52:**11–15.
215. **Urban, C. F., D. S. M. Ermert, U. Abu-Abed, C. Goosman, W. Nacken, V. Brinkmann, P. R. Jungblut, and A. Zychlinsky.** 2009. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathog.* **5:**e1000639.
216. **van der Meijden, W. I., H. Koerten, W. van Mourik, and W. C. de Bruijn.** 1988. Descriptive light and electron microscopy of normal and clue-cell-positive discharge. *Gynecol. Obstet. Investig.* **25:**47–57.

217. **Vazquez, J. A., J. D. Sobel, R. Demitriou, J. Vaishampayan, M. Lynch, and M. J. Zervos.** 1994. Karyotyping of *Candida albicans* isolates obtained longitudinally in women with recurrent vulvovaginal candidiasis. *J. Infect. Dis.* **170:**1566–1569.
218. **Vazquez-Torres, A., J. Jones-Carson, and E. Balish.** 1995. Nitric oxide production does not directly increase macrophage candidacidal activity. *Infect. Immun.* **63:**1142–1144.
219. **Villar, C. C., H. Kashleva, C. J. Nobile, A. P. Mitchell, and A. Dongari-Bagtzoglou.** 2007. Mucosal tissue invasion by *Candida albicans* is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. *Infect. Immun.* **75:**2126–2135.
220. **Vylkova, S., N. Nayyar, W. Li, and M. Edgerton.** 2007. Human beta-defensins kill *Candida albicans* in an energy-dependent and salt-sensitive manner without causing membrane disruption. *Antimicrob. Agents Chemother.* **51:**154–161.
221. **Vylkova, S., J. N. Sun, and M. Edgerton.** 2007. The role of released ATP in killing *Candida albicans* and other extracellular microbial pathogens by cationic peptides. *Purinergic Signal* **3:**91–97.
222. **Wagner, R. D., C. Pierson, T. Warner, M. Dohnalek, J. Farmer, L. Roberts, M. Hilty, and E. Balish.** 1997. Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. *Infect. Immun.* **65:**4165–4172.
223. **Webb, B. C., C. J. Thomas, M. D. Willcox, D. W. Harty, and K. W. Knox.** 1998. *Candida*-associated denture stomatitis. Aetiology and management: a review. Part 2. Oral diseases caused by *Candida* species. *Austral. Dent. J.* **43:**160–166.
224. **Weissenbacher, T. M., S. S. Witkin, A. Gingelmaier, C. Scholz, K. Friese, and I. Mylonas.** 2009. Relationship between recurrent vulvovaginal candidosis and immune mediators in vaginal fluid. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **144:**59–63.
225. **Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel.** 1989. Risk factors for hospital-acquired candidemia. A matched case-control study. *Arch. Intern. Med.* **149:**2349–2353.
226. **Wheeler, R. T., D. Kombe, S. D. Agarwala, and G. R. Fink.** 2008. Dynamic, morphotype-specific *Candida albicans* beta-glucan exposure during infection and drug treatment. *PLoS Pathog.* **4:**e1000227.
227. **White, M. H.** 1996. Is vulvovaginal candidiasis an AIDS-related illness? *Clin. Infect. Dis.* **22**(Suppl. 2)**:**S124–S127.
228. **White, S. J., A. Rosenbach, P. Lephart, D. Nguyen, A. Benjamin, S. Tzipori, M. Whiteway, J. Mecsas, and C. A. Kumamoto.** 2007. Self-regulation of *Candida albicans* population size during GI colonization. *PLoS Pathog.* **3:**e184.
229. Reference deleted.
230. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317.
231. **Witkin, S. S.** 1986. Inhibition of *Candida*-induced lymphocyte proliferation by antibody to *Candida albicans*. *Obstet. Gynecol.* **68:**696–699.
232. **Witkin, S. S., J. Hirsch, and W. J. Ledger.** 1986. A macrophage defect in women with recurrent *Candida* vaginitis and its reversal in vitro by prostaglandin inhibitors. *Am. J. Obstet. Gynecol.* **155:**790–795.
233. **Wormley, F. L., Jr., J. Chaiban, and P. L. Fidel, Jr.** 2001. Cell adhesion molecule and lymphocyte activation marker expression during experimental vaginal candidiasis. *Infect. Immun.* **69:**5072–5079.
234. **Wormley, F. L., Jr., C. Steele, K. Wozniak, K. Fujihashi, J. R. McGhee, and P. L. Fidel, Jr.** 2001. Resistance of T-cell receptor δ-chain-deficient mice to experimental *Candida* vaginitis. *Infect. Immun.* **69:**7162–7164.
235. **Wozniak, K. L., J. E. Leigh, S. Hager, R. K. Swoboda, and P. L. Fidel, Jr.** 2002. A comprehensive study of *Candida*-specific antibodies in saliva of HIV-infected persons with oropharyngeal candidiasis. *J. Infect. Dis.* **185:**1269–1276.
236. **Wozniak, K. L., G. Palmer, R. Kutner, and P. L. Fidel, Jr.** 2005. Immunotherapeutic approaches to enhance protective immunity against *Candida* vaginitis. *Med. Mycol.* **43:**589–601.
237. **Wozniak, K. L., F. L. Wormley, Jr., and P. L. Fidel, Jr.** 2002. *Candida*-specific antibodies during experimental vaginal candidiasis in mice. *Infect. Immun.* **70:**5790–5799.
238. **Wray, D., D. H. Felix, and C. G. Cumming.** 1990. Alteration of humoral responses to *Candida* in HIV infection. *Br. Dent. J.* **168:**326–329.
239. **Wuthrich, M., B. E. Finkel-Jiminez, and B. S. Klein.** 2000. Interleukin 12 as an adjuvant to WI-1 adhesin immunization augments delayed-type hypersensitivity, shifts the subclass distribution of immunoglobulin G antibodies, and enhances protective immunity to *Blastomyces dermatitidis* infection. *Infect. Immun.* **68:**7172–7174.
240. Reference deleted.
241. **Yano, J., E. Lilly, M. Barousse, and P. L. Fidel, Jr.** 2010. Epithelial cell-derived S100 calcium-binding proteins as key mediators in the hallmark acute neutrophil response during *Candida* vaginitis. *Infect. Immun.* **78:**5126–5137.
242. **Yano, J., E. Lilly, C. Steele, D. Fortenberry, and P. L. Fidel, Jr.** 2005. Oral and vaginal epithelial cell anti-*Candida* activity is acid-labile and does not require live epithelial cells. *Oral Microbiol. Immunol.* **20:**199–205.
243. **Yeater, K. M., J. Chandra, G. Cheng, P. K. Mukherjee, X. Zhao, S. L. Rodriguez-Zas, K. E. Kwast, M. A. Ghannoum, and L. L. Hoyer.** 2007. Temporal analysis of *Candida albicans* gene expression during biofilm development. *Microbiology* **153:**2373–2385.
244. **Zelante, T., A. De Luca, P. Bonifazi, C. Montagnoli, S. Bozza, S. Moretti, M. L. Belladonna, C. Vacca, C. Conte, P. Mosci, F. Bistoni, P. Puccette, R. A. Kastelein, M. Kopf, and L. Romani.** 2007. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur. J. Immunol.* **37:**2680–2682.
245. **Zimmer, D. B., E. H. Cornwall, A. Landar, and W. Song.** 1995. The S100 protein family: history, function, and expression. *Brain Res. Bull.* **37:**417–429.

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch11

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Innate Immunity to *Candida* Infections

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INTRODUCTION—SCENE SETTING

An understanding of the virulence and pathogenesis of a microorganism has to be framed in the knowledge of how the pathogen interacts with the human immune system. Ultimately it is the balance between the virulence attributes of the pathogen and the defensive capabilities of the host that determines the outcome of interactions between microbes and the human body. Pathogens that have evolved to be aggressively virulent and have the capacity to overcome a healthy immune system (e.g., some respiratory viruses and waterborne bacterial pathogens) also have to be highly transmissible to enable them to escape the hosts that have succumbed to infection. However, other organisms have evolved a more benign pathogenesis strategy—some tending to a more commensal relationship with their host, so that virulence is manifest only under the almost accidental circumstances of the highly immunocompromised patient setting. Infections due to *Candida* species are predominantly of the latter, although vaginitis and other superficial *Candida* infections can occur in fully immunocompetent women. However, even in the latter cases, infection can usually be traced to some clinical intervention or disturbance in the microbiological status quo, such as treatment with a broad-spectrum antibacterial antibiotic that upsets the normal balances that maintain this fungus in a commensal relationship with its host on the mucosal surface. It may be that host invasion occurs via mechanisms that have evolved primarily for the maintenance and survival of the fungus on the mucosa rather than systemic invasion of the host. For example, hypha infiltration of tissue may, under normal circumstances in the healthy host, be an adaptation for retention of the fungus on epithelial layers. Epithelial cells undergo sloughing and are washed in fluids, so the potential for the elimination of the organism from that environment is high. In this light, the penetration of the mucosa leading to systemic disease may occur when the surveillance and blocking of tissue invasion by the innate immune system are temporarily repressed, resulting in deeper-than-normal infiltration of the surface layers to an extent that enables the fungus to reach the bloodstream, from which it can be disseminated to the major organs.

Another feature of *Candida* disease, shared by mycoses caused by other fungi, including *Aspergillus fumigatus*, is that tissue pathology is sometimes due to pathogen-associated virulence factors (hypha formation, production of secreted hydrolases, etc.), while in other settings tissue damage is due mostly to host-driven tissue destruction brought about by hyperactivation of the immune inflammatory response (23). In systemic candidiasis, for example, a wide range of *Candida* virulence traits are expressed at different times as the infection progresses. This is reinforced by reports of large numbers of single gene mutations that result in attenuated virulence. Therefore, pathogenesis is related in part to a large number of differentially expressed virulence factors (20, 25). However, women who suffer from recurrent episodes of *Candida* vulvovaginal candidiasis (VVC) have been shown to differ from women who do not suffer from this condition in having greatly increased hypersensitivity to the fungus. This results in enhanced neutrophil infiltration and pathogenic inflammation in VVC patients (33). Therefore, for candidiasis disease pathology can be either pathogen dependent, host dependent, or both.

Recent research has seen a significant growth of interest and progress in understanding the immunology of *Candida* infections. The molecular components of the surface of the fungus that stimulate, modulate, and attenuate innate recognition and immune activation are being defined, and an ever-increasing number of leukocyte receptors that engage with these molecules and result in the activation of the innate, and subsequently adaptive, immune responses are being discovered. Genetic polymorphisms that correlate with fungal disease susceptibility are also being recognized, and these underline the importance of various immune recognition phenomena. This chapter explores this recent literature, which is motivated by the hope that an understanding of this dynamic process may enable future immunotherapies to complement chemotherapy to better control *Candida* disease.

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OVERVIEW OF INNATE IMMUNITY DEFINING THE CELL TYPES AND THE ACTIVATION OF THE ADAPTIVE IMMUNE RESPONSE

Host defense against infections in general, and infections with *Candida* spp. in particular, depends on rapid activation of an acute inflammatory response by innate immunity (hours to days), followed by an incremental stimulation of specific immune responses mediated by T lymphocytes (cellular immunity) or B lymphocytes (humoral immunity). The idea generally accepted by immunologists until the last 10 years was that, while effective, innate immunity is nonspecific and "rather primitive and dumb" (97). However, this simplistic model, in which innate immunity performs only simple "ingest and destroy" tasks, could not explain how innate immune cells recognize microbial pathogens as "non-self" and the mechanisms that mediate an acute inflammatory response that is tailored to the type of pathogen encountered. Moreover, recent years have witnessed a chain of discoveries that demonstrated a strong relationship between the initial innate immune response and the subsequent development of specific immune responses (110).

It has therefore become obvious that mechanisms must exist that enable the rapid recognition of the invading pathogen, leading to the initiation of inflammation and the subsequent stimulation of adaptive responses delivered by T cells and B cells through their interaction with antigen-presenting cells (especially dendritic cells [DCs]) (52). The tasks of recognition of the invading pathogen and host defense activation are accomplished by pattern recognition receptors (PRRs) that sense conserved chemical signatures of the microorganisms called pathogen-associated molecular patterns (PAMPs) (93). It is now clear that PRR engagement by PAMPs is a crucial event coordinating the succession of events leading to pathogen elimination. In the case of *Candida* infection, these events start with *Candida* phagocytosis and continue with *Candida* killing by phagocytes, stimulation of cytokines, and the induction of a T-cell-specific response.

Candida Phagocytosis

Phagocytosis of *Candida* is mediated by the concerted action of humoral and cellular components of the innate immune system. The humoral components of the innate host defense involved in antifungal defence are represented by complement and mannose-binding lectin (MBL). Complement binding and activation are mediated by the alternative pathway, and complement activation is important mainly for the chemotaxis and opsonization of the fungus but not for lysis, which is prevented by the thick and complex cell wall (71). Furthermore, although MBL does bind and recognize *Candida*, the lectin pathway of complement activation probably plays only a minor role in *Candida* uptake (72).

The most important cell populations involved in the phagocytosis (and subsequent killing) of fungal pathogens are neutrophils and macrophages, and together with the monocytes they have been shown to represent the major producers of proinflammatory cytokines. These innate immunity cell populations have subsequently been shown to be the most important cellular component of host defense against disseminated *Candida* infections. Several membrane-bound receptors have been reported to contribute to the phagocytosis of *Candida albicans* by phagocytes, among which C-type lectin receptors (CLRs) such as dectin-1 (49), the mannose receptor (MR) (30, 121), and the DC-specific ICAM3-grabbing nonintegrin (DC-SIGN) (21) play a major role, as described in more detail later. In contrast, CD11b/CD18 (named also complement receptor 3 [CR3]) has been proposed to be an important *Candida* phagocytic receptor in neutrophils (137) and eosinophils (159). The Toll-like receptors (TLRs) do not mediate fungal uptake, but they seem to be involved in directing the subsequent maturation of the phagosome and the presentation of antigens, as shown for microbial (but not yet fungal) pathogens (11).

The Killing of *Candida*

Following uptake by neutrophils and macrophages, killing of *Candida* occurs through both oxidative and nonoxidative mechanisms (120). The β-glucan receptor dectin-1 has been shown to induce the respiratory burst in response to fungi, an activity which can be enhanced by TLR signaling (36). The respiratory burst is an essential antifungal effector mechanism resulting in the production of toxic oxidants (4, 86) and activation of granule proteases capable of killing *Candida* (116). Dectin-1 has been reported to directly induce fungicidal mechanisms of neutrophils (64). Killing of *Candida* has also been shown to occur extracellularly, through the as-yet-undefined actions of PRRs such as galectin-3 (68).

Killing by *Candida*

Monocytes such as macrophages avidly take up *C. albicans* yeast cells, but hyphae are stimulated to form within the phagolysosome, and after a period, hyphal growth can rupture the monocyte membrane, thereby killing the monocyte. The ability of hyphae to kill macrophages in this way is highly attenuated in mutants that are impaired in the ability to form hyphae (84, 91). Normal cell wall phosphomannosylation is required for efficient recognition and phagocytosis, and defects in *C. albicans* O- and N-linked glycosylation result in increased rates of uptake into macrophages but decreased capacity to kill the macrophage (91). Arginine metabolism has been shown to be required for growth in and killing of macrophages and hence escaping host defense (56). Glycolipid-phospholipomannan fractions of *C. albicans* have been shown to be able to induce macrophage apoptosis (56, 57). *C. albicans* hyphae are more efficiently killed within neutrophils where there is insufficient time for germ tubes to form and disrupt the phagocyte (35, 158). Indeed, neutrophils were specifically attracted to hyphal filaments rather than yeast cells, a recognition process that is mediated by MEK/extracellular signal-regulated kinase (ERK) signaling. This suggests that neutrophils are able to detect and interact with hyphal cells and then kill them rapidly before hyphal elongation destroys the phagocyte (158). *C. albicans* has a greater capacity to destroy macrophages by physically rupturing such cells during hypha formation or by inducing apoptosis.

Cytokine Induction

One of the most important events occurring during the first hours of the host encountering the invading *Candida* is the release of proinflammatory mediators such as cytokines and chemokines. The release of these mediators is a coordinated event leading to recruitment of inflammatory cells at the site of infection, stimulation of the fungicidal effects of phagocytes, and subsequent activation of specific cellular responses (103). The PRRs and mechanisms responsible for the production of cytokines during the innate host response are described in detail below. One important aspect that has to be taken into account is the effect exerted by these innate

immune responses on the initiation of protective T-helper (Th)-dependent mechanisms.

Initiation of Th Adaptive Responses

In addition to the activation of the innate host defense mechanisms by *C. albicans*, an important process through which the innate immune system contributes to pathogen elimination is the initiation of adaptive immune responses. Antigen-presenting cells such as DCs, but also macrophages and B cells, ingest and process the pathogens and thereafter present them to naïve Th cells. This process is on the one hand amplified by costimulatory molecules and on the other hand directed by the production of a variety of proinflammatory cytokines that can induce differentiation towards specific Th subsets. It is generally accepted that induction of a Th1-dependent response that induces both cellular immunity and production of opsonizing antibodies is crucial for the defense against *C. albicans* (34). In contrast, a Th2 cellular response is considered nonprotective, as it induces class switch to nonopsonizing antibody subclasses and immunoglobulin E (IgE) (128).

Investigation of the role of Th17 in mediating the immune response has shown that Th17 memory cells are induced by *Candida* hyphae (1, 162), and in a murine model interleukin-17A receptor (IL-17AR) knockout mice had an increased susceptibility to systemic candidiasis (55). However, deleterious effects of IL-17 inflammatory activities have also been proposed in certain murine models (13, 160). On the other hand, patients with an impaired IL-17 production suffer from mucosal *C. albicans* infections in hyper-IgE syndrome and chronic mucocutaneous candidiasis (29, 96, 143). In contrast to Th cells, regulatory T cells (Tregs) suppress inflammatory responses in disseminated *C. albicans*, resulting in higher susceptibility in mice (108, 133). However, the tolerization-inducing effects of Tregs are likely to have beneficial effects at mucosal sites (147).

Summarizing these data, one could conclude that for optimal protection against (chronic) mucosal *Candida* infections a balanced response dependent on Th1, Th17, and Tregs is crucial. An effective Th1 (cellular) response and opsonizing antibody (humoral) response are important for defense against disseminated *Candida* infections. However, the most important effector mechanisms for host defense against disseminated candidiasis remain the cellular innate responses mediated by neutrophils and macrophages, as detailed above.

THE *CANDIDA* CELL WALL: AN IMMUNOLOGICAL SUPERMARKET

The cell wall of *Candida* is the point of contact between the fungus and the human host. Similarly, the molecules that constitute the wall are presented to and recognized by the immune system via series of receptor-ligand interactions. The fungal agonists and antagonists that bind to the immune cell receptors are termed PAMPs, and these engage with various PRRs, which include the TLRs and the C-type lectins.

Candida Cell Wall Structure

The cell wall of *Candida albicans* has been characterized in terms of its overall carbohydrate composition (66, 103) and the cell wall proteome (67). It is likely that the general structures of the walls are similar in many of the ascomycetous yeasts, from *Saccharomyces cerevisiae* to *C. albicans*, although not all features of the carbohydrate components are conserved and the cell wall proteome and complement of wall biosynthetic genes differ substantially among species (19, 58). The bulk of the wall is composed of an outer layer of mannoproteins (35 to 40%) and β-1,6 glucan linkers (20%) that attach these proteins to the inner skeletal layer, which is composed of β-1,3 glucan (40%) and chitin (2%) (Color Plate 3). The global composition of the wall does, however, change with environmental conditions; in particular, the chitin content is known to increase substantially in cells with damaged β-1,3 glucan (151). The outer cell wall layer mannoproteins are lollipop-shaped proteins with rod-like regions stabilized by O-linked glycans and an N-terminal globular domain that may be heavily N glycosylated. These glycoproteins are 80 to 90% sugar by molecular mass, and they include the *Candida* adhesins that are predominantly glycosylphosphatidylinositol proteins linked via β-1,6 glucan to the β-1,3 glucan–chitin skeleton. In *C. albicans* O-linked mannan is represented by an ether-linked linear oligosaccharide of up to five α-1,2 mannose sugars (100). The N-linked mannan is a much larger structure that is amide linked to asparagine residues of the polypeptide. *N*-Mannan compromises a triantennary $Man_8GlcNAc_2$ complex that is well conserved between lower and higher eukaryotes and a fungus-specific outer-chain mannan of up to 150 mannose sugars built as a series of α-1,2, β-1,2, and α-1,3 mannose side chains that are attached to an α-1,6 backbone (103). A fraction of the *N*-mannan can be hydrolyzed in mild acid due to the disruption of phosphodiester bonds that attach β-1,2-linked phosphomannosaccharides (phosphomannan) to *N*-mannan. Recent work has shown that all of the major glycans of the cell wall participate in immune recognition by engaging a range of PRRs in a variety of immune cell types. Mannan structure and size are variable and can be influenced by a range of environmental stresses (70).

Recognition of the Cell Wall

A great deal of recent research has focused on the components of the cell wall that are recognized by PRRs (Color Plate 3). These studies are discussed in detail below, but the broad picture is that each of the major components of the cell wall has now been identified or implicated as being immunologically relevant (103, 142). *N*-Mannans interact with the mannose receptor C-type lectin on monocytes and with DC-SIGN of DCs (21, 106), while O-mannan is an agonist of TLR4. Hyphal *N*-mannan is recognized by dectin-2 (126), and CR3 is able to recognize both mannan and β-glucan fractions (16). The galectin-3 receptor is involved in the sensing of β-1,2 mannose residues of *N*-mannan (61), but no cytokines are induced by exposure to phosphomannan (106). The latter does have a role, however, in mediating uptake into macrophages (91). β-1,3 glucan is the major ligand for the lectin dectin-1 and TLR2 (17, 18, 27, 36, 42, 135), while β-1,6 glucan seems to act as an opsonic phagocytic receptor for neutrophils (122). Chitin—a β-1,4-linked homopolymer of *N*-acetylglucosamine—has not yet been fully investigated as a PAMP, but it has been shown to cause basophil, eosinophil, and neutrophil accumulation in tissues and alternative macrophage activation associated with helminth immunity (115). The immune reactivity of chitin depends on the size of the chitin presented as a PAMP, and the receptor for chitin binding has yet to be unequivocally identified (26, 77). Crustacean chitosan has also been implicated in the inactivation of DCs via TLR4 (149). *Candida* also has cell wall glycolipid composed of mannose-inositol-phosphoceramide (phospholipomannan) that also

plays a role in immune recognition via binding to TLR2 (62).

Masking and Unmasking of β-1,3 Glucan

In addition to being the dominant polysaccharide in the C. *albicans* cell wall, β-1,3 glucan is also one of the most proinflammatory. Under normal conditions this skeletal component of the wall is masked from the immune system by the mannoprotein outer cell wall layer. Mutations in cell wall glycosylation and heat treatment of the cell remove or permeabilize this mask and expose β-1,3 glucan to dectin-1 (42, 154). Also, hyphal cells lack bud scars, which are one of the few places on the cell where β-1,3 glucan is exposed at the cell wall surface. It should be remembered that the superficial mannans are in themselves immunostimulatory but that exposure of the β-1,3 glucan–dectin-1 recognition system results in the recognition system shifting from that biased towards mannan recognition to the detection of β-1,3 glucan. During infection it has also been shown that β-1,3 glucan is initially masked but becomes unmasked later on during infection (155). Exposure to β-1,3 glucan inhibitors such as caspofungin has also been shown to lead to unmasking of the β-1,3 glucan recognition system in both C. *albicans* (155) and A. *fumigatus* (53, 74). Treatments with antifungal agents other than echinocandins have also been shown to modulate the immune response, probably due to primary or secondary effects of the antifungal drugs on the cell wall structure (10).

PRRs FOR *CANDIDA*

During an infection with *Candida* spp., the initial response of the innate immune system will be determined by the recognition of fungal cell wall components. In the case of C. *albicans*, both mannans and mannoproteins that cover the fungus are recognized by specific PRRs, as well as β-glucans and chitin at the level of the budding scar (37). These structures represent the initial fungal PAMPs recognized as nonself by receptors on the surface of mainly myeloid cells: the so-called PRRs. Several classes of PRRs have been described during the last few years: the TLRs, the CLRs, the nucleotide binding domain leucine-rich repeat-containing receptors (NLRs), and the RigI helicases (3). While RigI helicases have been mainly involved in the recognition of viruses, all the other three families of PRRs have been implicated in recognition of *Candida* PAMPs and induction of innate host responses.

Toll-Like Receptors

The first suggestion for a fundamental role for TLRs in antifungal host defense was made by Lemaitre and colleagues, who observed that *Drosophila* flies deficient in the Toll receptor rapidly succumbed to A. *fumigatus* infection (80). Mammalian TLRs have a leucine-rich repeat, extracellular domain that is responsible for the recognition of microbial or fungal structures and share a cytoplasmic Toll/IL-1 receptor domain with IL-1 and IL-18 receptors, responsible for transducing intracellular signals (118). Ligand recognition by TLRs and transduction of intracellular signals by several adaptor proteins, such as MyD88, Mal, TRIF, and TRAM, induce the activation of kinase cascades, eventually resulting in activation of transcription factors, such as NF-κB, NF-AT, and IRF3, that induce gene expression and production of various chemokines and cytokines (3). Shortly after the discovery of TLRs, TLR2 and TLR6 were shown to be involved in the recognition of the fungal structure zymosan, which is derived from *Saccharomyces cerevisiae* (112). Moreover, the adaptor molecule MyD88, which is shared by most TLRs, has been proven to be crucial for antifungal defense by several in vivo studies (9), strongly suggesting that TLRs play a crucial role in host defense against fungi.

TLR2

One of the first studies that investigated TLR2 in fungal host defense reported that blocking of TLR2 by specific antibodies resulted in decreased production of tumor necrosis factor (TNF) and IL-1β after stimulation of monocytes by C. *albicans* (104). Shortly thereafter it was proposed that TLR2 from myeloid cells recognizes the phospholipomannan component of the *Candida* cell wall (62), which was later confirmed in keratinocytes (82).

Several ensuing studies have assessed the functional effects of TLR2 during *Candida* infections. In vitro studies have shown that TLR2$^{-/-}$ mice have decreased production of TNF-α and MIP-2 and reduced neutrophil recruitment after a challenge with *Candida* (148) and that TLR2-mediated recognition of C. *albicans* leads to a proinflammatory Th1 host response (102). However, two other studies found that TLR2$^{-/-}$ mice showed an increased resistance to disseminated candidiasis that was accompanied by decreased production of IL-10 and increased IL-12 and gamma interferon (IFN-γ) production (9, 108). In line with this, TLR2-deficient macrophages have been shown to have an increased ability to contain C. *albicans* (12). These immunomodulatory effects induced by TLR2 were found to be mediated through the generation of Tregs with immunosuppressive potential (108, 133). An anti-inflammatory role for TLR2 in antifungal host defense is further supported by a recent study reporting that zymosan can tolerize DCs through a TLR2 and dectin-1-mediated pathway involving mitogen-activated protein kinase (MAPK)/ERK (28). A limited role for TLR1 and especially TLR6, two receptors known for forming heterodimers with TLR2, has been recently reported in the case of C. *albicans* recognition (109).

In conclusion, TLR2 is involved in the recognition of phospholipomannan of C. *albicans*, although disagreement sometimes persists regarding its functional effects. Overall, the general consensus is that TLR2 ligands induce weaker proinflammatory effects than TLR4 ligands (50). In addition, TLR2 has also been shown to have immunosuppressive effects on anti-C. *albicans* host defense through promoting environments that favor Th2 or Treg-type responses (108, 133).

TLR4

TLR4 is one of the most intensely studied PRRs, due to its role as the main receptor of bacterial lipopolysaccharides (114). TLR4 is very potent in mediating proinflammatory responses, and this occurs through MyD88-Mal-induced activation of NF-κB (111), production of IL-12 through activation of the p38/Jun N-terminal protein kinase pathway (2), and induction of IRF3 through TRIF-TRAM signaling leading to type I interferon secretion (136). In addition to recognition of lipopolysaccharide, Tada et al. have reported for the first time that TLR4 recognizes mannans from S. *cerevisiae* and C. *albicans* (134). A subsequent study found that short linear O-linked mannans of C. *albicans* are recognized by TLR4 and induce proinflammatory cytokines such as TNF-α (106).

In a first experimental infection model of disseminated C. *albicans* infection, it has been shown that the absence of TLR4-mediated signaling in mice resulted in decreased

chemokine production and impaired neutrophil recruitment (104). However, other studies have observed variable results, with TLR4$^{-/-}$ mice either being more susceptible in models of intragastric infection or intravenous reinfection (9), being not different from wild-type animals in models of intravenous infection with *Candida* yeasts (101), or even surviving longer in a model of intravenous infection with *C. albicans* hyphae (9). Differences among the experimental models and/or the *C. albicans* strains are believed to be responsible for these differences. In line with this, a recent survey of *C. albicans* strains from various genetic clades showed that only some of the strains were recognized by TLR4 (105).

Overall, TLR4 appears to participate in antifungal host defense by recognizing mannan structures and mediating proinflammatory responses. A recent study in a system of human neutrophils and epithelial cells has also demonstrated an important role of TLR4 for the anti-*Candida* protection of epithelial cells (152).

TLR9

Unmethylated CpG sequences are the natural ligands for TLR9, and several reports have now suggested that TLR9 can recognize fungal DNA. Indeed, TLR9 is able to recognize fungal DNA from *C. albicans* and upon recognition induces stimulation of cytokines in DCs (98). Similarly, blocking of TLR9 in human monocytes and TLR9-deficient mouse macrophages stimulated with *C. albicans* leads to a reduced production of cytokines, mainly IL-10 (145). However, Bellochio et al. have reported that TLR9$^{-/-}$ mice produced less IL-12 and more IL-4 and IL-10, but this had little effect on the overall mortality rate of the animals (9). The redundant effect of TLR9 for anti-*Candida* host defense was supported by two additional studies (98, 145). In conclusion, most of the data available at this time suggest a role for TLR9 for the recognition of fungal DNA, but the magnitude of this effect for the overall antifungal defense is likely to be overshadowed by redundant signals induced by other PRRs.

C-Type Lectin Receptors

CLRs are members of a large family of PRRs including dectin-1, the macrophage MR, DC-SIGN, dectin-2, and the circulating MBL. These receptors share one or more carbohydrate recognition domains that were originally found in the MBL (161) and are involved in the recognition of polysaccharide structures from microorganisms but also endogenous ligands. Importantly, over the recent years these receptors have been shown to be central for the fungal recognition and the induction of the innate immune response.

Dectin-1

Dectin-1 recognizes β-1,3 glucans in a calcium-independent way and is involved both in ligand uptake and phagocytosis, as well as proinflammatory cytokine production (15). Dectin-1 signals through the kinase Syk and the adaptor CARD9, and this pathway has been shown to induce IL-2 and IL-10 in DCs (79). Leibundgut-Landmann et al. have also demonstrated that infection with *C. albicans* induces protective dectin-1/CARD9-dependent Th-17 responses that have a key role in fungal host defense (79). In addition to these studies in murine cells, cytokine production induced by *C. albicans* by human peripheral blood mononuclear cells has been shown to be dependent on dectin-1 (42). Dectin-1 intracellular signaling is mediated either through CARD9 (119) or through the noncanonical Raf1 pathway (43). Although dectin-1 signaling alone is sufficient to induce responses upon fungal recognition, several studies have emphasized that dectin-1 cooperates with TLRs, leading to synergistic proinflammatory responses. Two independent studies have shown that dectin-1 in collaboration with TLR2 triggers proinflammatory responses upon stimulation with *C. albicans* and zymosan (18, 36). Recently, dectin-1 has been found to amplify TLR4-dependent pathways in both murine (27) and human (32) myeloid cells. In contrast, dectin-1 signaling and cytokine induction are inhibited by the tetraspanin CD37 (146).

The in vitro studies on the role of dectin-1 for recognition of *Candida* have been complemented by in vivo studies demonstrating the important role of this receptor for antifungal host defense. It has also been shown that dectin-1$^{-/-}$ mice are more susceptible to infection with *C. albicans*, resulting in lower survival rates and increased fungal burdens (135). However, another study using a different strain of dectin-1 knockout mice could not confirm this for *C. albicans* infection but found an increased susceptibility to *Pneumocystis* infection (125). On the other hand, the adaptor molecule CARD9, involved in the dectin-1 signaling pathway, has been demonstrated to have a crucial role in survival during disseminated candidiasis (45). Overall, these data suggest an important role for dectin-1 in antifungal immunity, either directly or through collaborative signaling with TLR2 and/or TLR4. Its main role seems to be played in myeloid cells such as monocytes and macrophages, while in human neutrophils it has been proposed to play a secondary role, the main β-glucan receptor in these cells being CD11b/CD18 (CR3) (137).

Macrophage MR

One of the first identified fungal PRRs was the MR (129). MR has various domains that can recognize oligosaccharides terminating in GlcNAc (chitin is a polymer of GlcNAc), fucose, and mannose (130). The MR has been previously implicated in the recognition of *C. albicans* (87), and recently the role of the MR in the recognition of *C. albicans* has been strengthened by a study showing that it recognizes branched *N*-bound mannans from *C. albicans* (106). Like dectin-1, the MR has been initially suggested to play an important role in phagocytosis, although this has been questioned recently (76). In contrast, the MR has been proposed to be recruited relatively late to the phagosome after ingestion of *C. albicans* and to mediate intracellular signals leading to cytokine production (48). In line with this, a recent study has demonstrated an important role of the MR for the induction of protective Th17 responses by *C. albicans* (144).

In vivo data for mice defective for the MR are limited. Although one study suggested only a minor role for the MR in the host defense against *Candida* infections (78), this study employed an intraperitoneal model of infection with relatively little relevance to the clinical situation. More in vivo studies are needed before a definitive conclusion regarding the role of the MR in the in vivo situation of a *Candida* infection can be drawn.

Other CLRs

Dectin-2 is also a member of the CLR family and is mainly present on myeloid cells and maturing inflammatory monocytes (5). Dectin-2 recognizes high-mannose structures (90) and interacts with the FcgR to induce TNF in response to *C. albicans* hyphae (126). Therefore, dectin-2 mainly seems to play a role in hyphal recognition and is the first receptor described to produce proinflammatory cytokines in response

to fungal hyphae. Recently it has been shown that dectin-2 has important Th17-inducing activities during *Candida* infection (117).

DC-SIGN is primarily expressed on mature DCs and recognizes high-mannose structures in a calcium-dependent way (69). DC-SIGN mediates uptake of fungal particles and recognizes mannans from *C. albicans* (21, 22). Immunosuppressive effects through stimulation of IL-10 production have been suggested (38), but this remains to be demonstrated in fungal infections.

Finally, several other CLRs have recently also been suggested to contribute to anti-*Candida* host defense, such as Mincle (153) and the CD36/Scarf scavenger receptors (92). Galectin-3 is a receptor mainly expressed by macrophages and shown to be involved in the recognition of the β-mannosides of *C. albicans*, in close collaboration with TLR2, especially at the level of the intestinal mucosa (59, 61). MBL is a soluble CLR that is secreted by the liver and which can bind to *C. albicans* (65). MBL is mainly involved in fungal host defense due to its ability to opsonize fungal yeasts by activating the complement system (14).

Nucleotide Binding Domain Leucine-Rich Repeat-Containing Receptors

In addition to the mainly cell membrane-bound TLRs and CLRs, mammalian host defense has built a second line of recognition receptors, located intracytoplasmatically, that are able to trigger innate immune mechanisms upon the entrance of the pathogen in the cell. Two main classes of intracytoplasmatic PRRs have been described to date: RigI helicases and NLRs. While RigI helicases are mainly receptors for viruses, NLRs have two important functions for the host defense against cellular pathogens: recognition of bacterial peptidoglycans and activation of the inflammasome. Recognition of bacterial peptidoglycans is mediated by NOD1 and NOD2 receptors, which recognize muramyl peptides from the peptidoglycans of gram-negative and gram-positive bacteria, respectively (39, 40). These receptors play no role in the recognition of *C. albicans* (138).

In contrast to NOD1 and NOD2, an important role of the NLRP3 inflammasome for anti-*Candida* host defense has been proposed by some, though not all, studies. The inflammasomes are protein platforms that upon recognition of a microbial PAMP or an endogenous danger signal (e.g., ATP or uric acid) induce activation of a cysteine protease caspase-1, which, in turn, processes pro-IL-1β and pro-IL-18 into the bioactive cytokines (89). Both IL-1α- and IL-1β-deficient mice show increased mortality rates, and endogenous IL-1α and IL-1β are required for the induction of protective Th1 responses in disseminated candidiasis (150). IL-1β also proved to be important for polymorphonuclear leukocyte recruitment and generation of superoxide production (150). IL-18 is essential for differentiation of Th1 responses and secretion of IFN-γ, an essential component of antifungal host defense (132).

Surprisingly, however, caspase-1-deficient mice have been reported to have a normal resistance to disseminated candidiasis (95), suggesting activation of IL-1β by inflammasome-independent mechanisms. However, NLRP3$^{-/-}$ and ASC$^{-/-}$ mice have been reported to be more susceptible to both systemic (46, 73) and mucosal (51) *Candida* infections, opening the intriguing possibility of biological functions of inflammasome components that are not related to caspase-1 activation. Indeed, an earlier study on the function of ASC has reported its interaction with NF-κB and an influence on gene transcription (47). Whether ASC and NLRP3 have underestimated roles that are independent of inflammasome activation remains to be studied. However, in contrast to mouse macrophages that need activation of the inflammasome by *C. albicans* (60), caspase-1 is constitutively activated in human monocytes and thus does not need fungal recognition by the NLRs in the inflammasome (107). In conclusion, while the role of IL-1 and IL-18 for host defense against *Candida* infections is indisputable, the roles of the NLRP3 inflammasome for activation of these cytokines seem to differ depending on the cell type and host (mouse or humans), and additional studies are awaited to clarify these differences.

Cross Talk between PRRs

While many studies have described the various receptor systems that contribute to the recognition of *Candida* spp., it has become clear that an intense interaction between the pathways induced by these receptors is necessary for an optimal antifungal host response. Thus, recognition of *C. albicans* by monocytes and macrophages has been shown to be mediated by at least four recognition systems that sense fungal PAMPs of the *C. albicans* cell wall: recognition of N-linked mannans by the MR, recognition of O-linked mannans by TLR4, recognition of β-glucans by dectin-1/TLR2, and recognition of β-mannosides by galectin-3/TLR2 complexes (106). To these systems, recognition of chitin by a still-unidentified receptor, and possibly the recognition of hyphal fungal components by NLRP3, is likely to be added in the antifungal recognition armamentarium. If the fungal cell wall is able to trigger many different PRRs at the same time, it is important to realize that it is a complex interaction between the various pathways that ultimately leads to the host response.

Several interactions between PRRs are well documented. As mentioned earlier, dectin-1 is able to augment TLR2-mediated MAPK and NF-κB pathways leading to proinflammatory responses (18, 36) and to amplify TLR4 responses through a Syk-dependent pathway (27). Galectin-3, a PRR which recognizes β-(1,2) mannosides, has recently been shown to associate with TLR2, and this leads to the discrimination between the pathogenic *C. albicans* and the nonpathogenic *S. cerevisiae*. In addition, the TLR2 pathway itself is able to inhibit TLR4-mediated production of IL-12 through stabilization of c-Fos (2). Further, when TLRs activate NF-κB, *C. albicans* can induce DC-SIGN-dependent signals which subsequently lead to acetylation of the NF-κB subunit p65, leading to an increased IL-10 production that shifts the balance towards a more anti-inflammatory response (44). These observations imply that cross talk between PRRs is essential to the complexity and flexibility of the innate immune response against *Candida* infections (Fig. 1).

MECHANISMS OF ESCAPE FROM INNATE IMMUNITY

Although innate immune recognition results in a very efficient host response, several reports have suggested that *Candida* sometimes escapes recognition from PRRs, and even uses these receptors to its benefit. One important mechanism through which *Candida* can escape the immune system is shielding of fungal PAMPs that could elicit an adequate immune response. Thus, the hyphal forms of *C. albicans* induce a more anti-inflammatory profile than do the yeast forms, suggesting that morphogenetic changes can modulate the immune response to the advantage of the fungus (141). It has been suggested that this is due to the

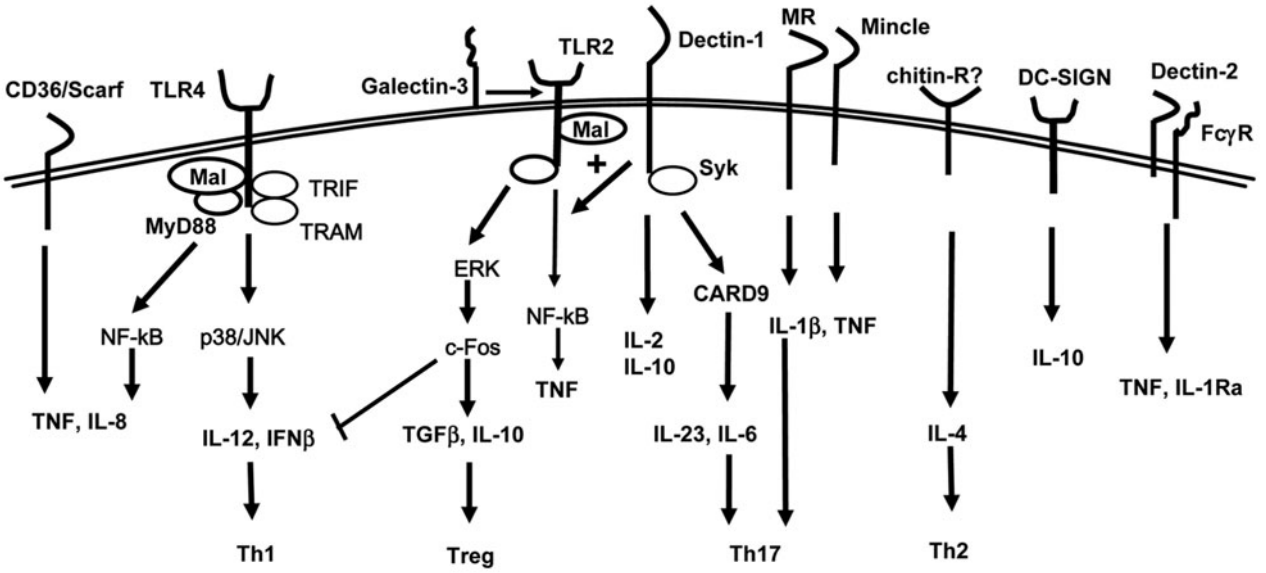


FIGURE 1 PRR recognition of *C. albicans* at the membrane level. Recognition of *C. albicans* at the level of cell membrane is mediated by TLRs and CLRs TLR4 induces mainly proinflammatory signals in monocytic cell types (monocytes, macrophages, and DCs) through the MyD88/Mal-mediated NF-κB and MAPK pathways while stimulating Th1 responses through IRF3-dependent mechanisms. TLR2 stimulates moderate amounts of proinflammatory cytokines while inducing strong IL-10 and transforming growth factor β (TGFβ) responses. On the one hand, this leads to the induction of a tolerant phenotype in DCs through an ERK/MAPK-dependent mechanism. On the other, TLR2 engagement induces proliferation of Tregs and immunosuppression. The proinflammatory effects of TLR2 can be amplified by dectin-1 and galectin-3, the latter especially in the macrophages. In addition to the amplification of TLR2 effects, the nonclassical lectin-like receptor dectin-1 induces IL-2, IL-10, and Th17 responses through a Syk/CARD9 cascade, independently of its interaction with TLR2. The classical lectin-like receptor MR induces proinflammatory effects in monocytes and macrophages, whereas chitin-dependent stimulation induces mainly Th2 responses, although this effect still has to be demonstrated for *C. albicans*. The identity of its receptor is unknown. Other less-well characterized pathways include stimulation of TNF and IL-1Ra by dectin-2, Mincle, and CD36/Scarf lectin receptors and of synthesis of the immunosuppressive cytokine IL-10 by DC-SIGN in DCs. 10.1128/9781555817176.ch11f1

shielding of the β-glucans from the surface of hyphae by the mannan layer in the outer portions of the cell wall (see above) (37). Several studies, as described above, have shown that activation of TLR2 can induce immunomodulatory signals, leading to a tolerogenic DC profile (28) and proliferation of Tregs (133). Furthermore, CR3 and FcgRII/FcgRIII have inhibitory effects on the activation of the immune system (121). Understanding these specific evading strategies that interfere with successful clearance of the fungal pathogen can guide the way for the development of novel therapeutic approaches.

POLYMORPHISMS OF INNATE IMMUNITY GENES AND PATIENT SUSCEPTIBILITY TO *CANDIDA* INFECTIONS

Although the in vitro and the experimental studies described above strongly suggest that PRRs are involved in the host defense against fungal pathogens, the most important challenge is to provide direct evidence of the role of PRRs for fungal infections in humans. Part of this may be achieved by experiments in which human primary cells are triggered with fungal microorganisms. However, valuable information can also be gathered from immunogenetic studies, in which the susceptibility to fungal infections in individuals bearing polymorphisms in the genes coding for the PRRs can be assessed.

Studies of patients with clinical conditions such as hyper-IgE syndrome and chronic mucocutaneous candidiasis, a familial syndrome characterized by severe oropharyngeal candidiasis and chronic onychomycosis, have identified defects in adaptive immunity (*AIRE* gene) and diminished Th17 responses as the cause of the disease (54) (Fig. 2). In contrast, the Asp299Gly TLR4 polymorphism has recently been identified as a susceptibility trait for systemic candidiasis, supporting a role for TLR4 in disseminated candidiasis (139). In contrast, no role of this polymorphism for vaginal colonization with *Candida* spp. (99) or in chronic mucocutaneous candidiasis (140) has been observed. The Asp753Gln TLR2 polymorphism resulted in an altered cytokine profile in patients with *Candida* sepsis, but a definitive role for susceptibility to candidiasis could not be determined due to the low number of patients (157). TLR1 functional polymorphisms also seem to play an important role in susceptibility to disseminated candidiasis (T. Plantinga and M. G. Netea, personal communication). Studies of additional functional polymorphisms in larger cohorts of patients are warranted in order to confirm the role of the

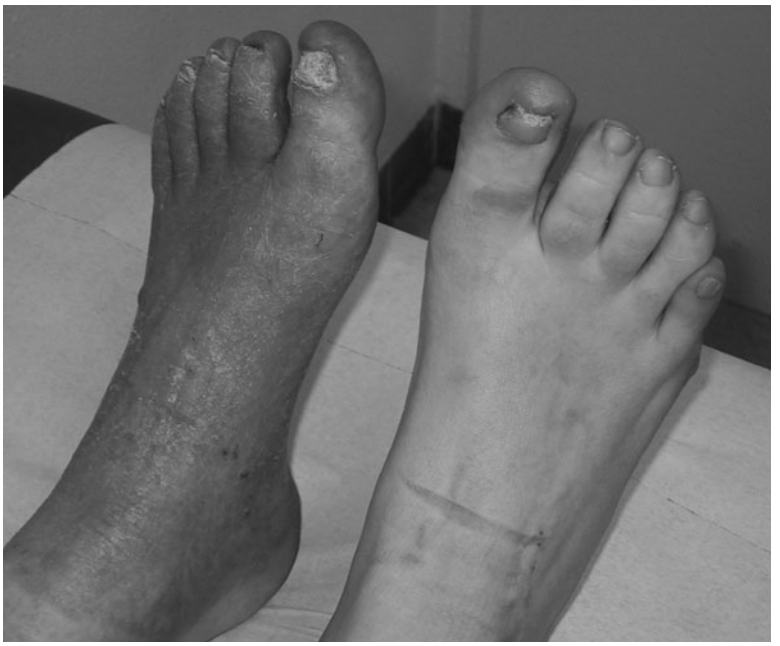


FIGURE 2 Extent of onychomycosis and dermatophytosis in a chronic mucocutaneous candidiasis patient with severe Th17 deficiency. 10.1128/9781555817176.ch11f2

various PRRs in the pathogenesis of invasive *Candida* infections.

Recently, the importance of dectin-1 in the sensing of *Candida* in human infections was confirmed (31). In a family with multiple subjects suffering from recurrent mucocutaneous fungal infections, the early stop codon polymorphism Y238X was discovered in dectin-1. This polymorphism led to the loss of the last 10 amino acids of the extracellular carbohydrate recognition domain involved in the binding of fungus-derived β-glucan. Defective surface expression of dectin-1 due to the presence of the Y238X polymorphism resulted in a lack of β-glucan recognition and impaired cytokine responses (IL-6, TNF, and IL-17) by monocytes and macrophages. In contrast, neutrophils of affected patients exhibited phagocytosis and normal killing of *C. albicans*. This underlines the redundant nature of dectin-1 in the phagocytosis and killing of yeast pathogens by human myeloid cells, explaining the absence of invasive candidiasis in these patients. The defective function of myeloid cells with regard to cytokine release in the patients bearing the Y238X dectin-1 polymorphism is the most likely cause of the clinical phenotype. However, defective dectin-1 signaling in epithelial cells and intraepithelial γ/δ T cells could also have contributed to the clinical picture, especially since these cells express dectin-1 and produce cytokines and antimicrobial peptides upon activation (88, 123). The role of dectin-1 for mucosal anti-*Candida* defense has been confirmed in a study showing that individuals bearing the dectin-1 stop polymorphism and undergoing stem cell transplantation are more likely to be colonized with *C. albicans* and more often need antifungal therapy (113). In addition, the role of the dectin-1 pathway for antifungal host defense has been also supported by the identification of a family bearing mutations in CARD9, the adaptor molecule mediating intracellular signaling induced by dectin-1. These patients also exhibited increased susceptibility to fungal infections (41).

A limited number of other studies have investigated the role of genetic variability of the host for susceptibility to recurrent vulvovaginal candidiasis (RVVC). A decrease in circulating MBL concentrations correlates with the presence of mutant alleles in exon 1 of the MBL gene. Babula and colleagues reported an association between RVVC and carriage of the Gly54Asp polymorphism of *MBL*, and the variant allele was also related to a reduction in the vaginal concentration of MBL (6). Other polymorphisms in exon 1 codons of the MBL gene have been identified and are also associated with reduced circulating MBL concentrations (83). It remains to be established whether these polymorphisms are also associated with an increased risk of RVVC.

IL-4 is one of the most important anti-inflammatory cytokines, and a high capacity for production of IL-4 has been associated with immunosuppressive effects. Local vaginal IL-4 levels were higher and median nitric oxide (NO) levels were lower in patients with RVVC than in controls (7). A single nucleotide polymorphism consisting of a cytosine-to-thymine variation in the promoter region of the IL-4 gene (C589T) has been reported to be associated with a 12-fold increase in the vaginal concentration of IL-4 and susceptibility to RVVC (7).

Vulvar vestibulitis syndrome (VVS), also known as vulvodynia, consists of an intense pain syndrome confined to the vaginal vestibule, which is defined as erythema and pain during introduction of a vaginal tampon, while touching the vagina with a cotton swab, and during gynecological examination or sexual intercourse. The causes of this condition and possible therapy have not yet been clarified, but a relationship between VVS symptom initiation and vaginal infection with *Candida albicans* has been proposed (156). In 2009, Lev-Sagie and colleagues performed a study evaluating the relationship of an NLRP3 gene length polymorphism and RVVC in 143 women with VVS and 182 control patients with no history of VVS. The *NLRP3* gene presents

four different tandem sequence repetition alleles; furthermore, those alleles could be combined into 10 different genotypes. In both VVS patients and control subjects, the homozygosity for the 12 allele repetitions in the intron was the most prevalent, whereas the presence of 6 allele repetitions was the least prevalent. The 12,12 genotype was found in a higher percentage of control subjects than in patients with RVVC and VVS. On the other hand, the 7-repetition allele was more frequently present in patients with VVS than in control subjects. It has been hypothesized that the possession of allele 7, which has 200 bp less than allele 12, may lead to the formation of an altered NLRP3-containing inflammasome or an inflammasome with a reduced biological activity (81). However, the relationship between VVS and *NLRP3* length polymorphism has not yet been completely clarified, and a potential association between *NLRP3* length polymorphism and RVVC in women who do not have VVS remains to be evaluated.

In conclusion, increasing evidence has emerged that has linked genetic polymorphisms of genes of innate immunity with *Candida* infections (Fig. 3). However, systematic genetic studies have yet to be performed for the major clinical syndromes involving *Candida* infections (systemic candidiasis and RVVC) in order to be able to evaluate properly the extent of the role of the genetic background in susceptibility to *Candida* infections.

THE FUTURE PROMISE OF ADJUVANT IMMUNOTHERAPY FOR *CANDIDA* INFECTIONS

Recent progress in understanding host defense against fungal infections in general, and *Candida* infections in particular, has provided important novel targets for potential novel immunotherapeutic approaches. As vaccination is one of the most cost-effective treatment strategies and probably the most powerful tool to protect humans and animals against infectious disease, further vaccine development could significantly lessen the burden of *Candida* infections in patients at risk. Development of a vaccine against *Candida* spp. should induce both cellular and humoral immune responses. Understanding the innate immune pathways leading to specific adaptive responses will enable the clinician to tailor vaccination to the type of infection for which the patient is at risk.

The discoveries of the last decade have shown that while Th1 responses are mainly responsible for the protective adaptive immunity during systemic candidiasis, the Th17 responses are mainly responsible for the mucosal anti-*Candida* defense. As the TLRs are strong inducers of inflammatory responses that mainly drive Th1 responses, and CLRs have the potential to modulate mainly Th17 immunity, one could envisage vaccination strategies based on adjuvants that

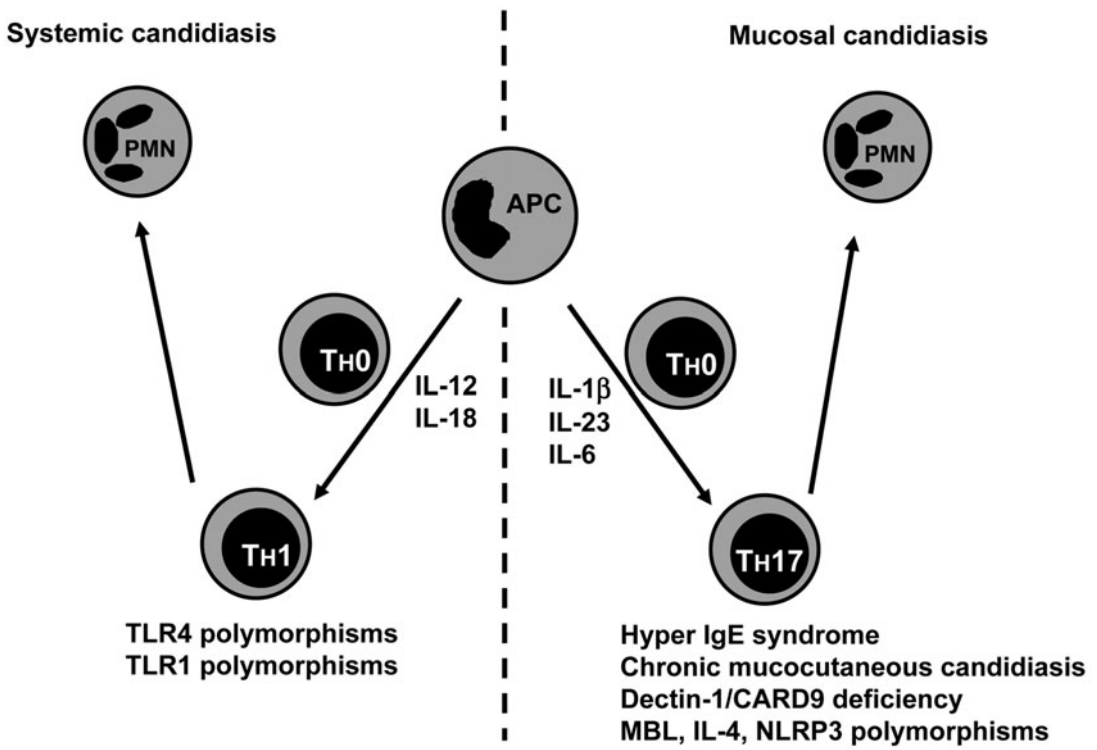


FIGURE 3 Genetic susceptibility to fungal infections. Host defense against both systemic and mucosal infections relies significantly on the proper function of neutrophils, explaining the high incidence of both types of fungal infections in neutropenic patients. However, an important difference seems to be present in terms of Th cell population responsible for activation of neutrophils in systemic candidiasis (Th1 cells) or mucosal candidiasis (Th17 cells). An increased susceptibility to systemic candidiasis is seen in patients with TLR4 or TLR1 polymorphisms. In turn, defects of IL-17 production such as in patients with hyper-IgE syndrome, patients with chronic mucocutaneous candidiasis or dectin-1/CARD9 deficiency, or patients bearing polymorphisms of MBL, IL-4, or NLRP3 are associated with an increased susceptibility to mucosal forms of *Candida* infections. 10.1128/9781555817176.ch11f3

would specifically induce either Th1 or Th17 responses, depending on the type of infection (either systemic or mucosal) for which a certain patient is at risk.

In addition to vaccination, recent insight in the pathophysiology of systemic candidiasis has led to the suggestion of adjuvant immunotherapy based on recombinant cytokine treatment. IFN-γ is a key cytokine for innate as well as acquired resistance to candidiasis, and administration of IFN-γ to mice infected with *C. albicans* has had a beneficial effect on outcome (131). The important role of endogenous IFN-γ in resistance against both gastrointestinal and systemic candidiasis has also been demonstrated by the increased susceptibility of knockout mice deficient in IFN-γ or IFN-γ receptors to infection with *Candida* (8, 24, 63, 75). Case reports have shown beneficial effects of IFN-γ in combination with antifungal drugs in treating a small series of hematologic patients with candidiasis (124), as well as a patient with an *Aspergillus fumigatus* brain abscess (127), and a patient with *Staphylococcus aureus* liver abscess and invasive *C. albicans* infection (85). Moreover, a recent small study using recombinant granulocyte-macrophage colony-stimulating factor has provided the proof of principle that adjuvant immunotherapy with recombinant cytokines can reverse immunoparalysis in sepsis and potentially improve the outcome of infection (94).

In conclusion, the basic and pathophysiological research of the last 20 years has provided a fundamental design of immunotherapeutic strategies in *Candida* infections. We are at the dawn of a new phase in translational and clinical research that will result in the translation of basic immunological knowledge into novel treatments that are aimed at improving the outcome for and quality of life of patients suffering from fungal infections.

CONCLUSIONS

Candida immunology has made significant strides in the last decade through the definition of many of the PAMP-PRR interactions using methods that combine the technologies and reagents within the medical mycology and immunology communities. It has become clear that the innate recognition of *Candida* cells involves a wide range of receptor-ligand interactions. We are, however, now defining new questions regarding the differences between the mannans and cell wall structures of different organisms and the nature of the receptors to certain polysaccharides such as chitin, and are piecing together the data that have been mined from ex vivo and in vitro experiments into the much more complex context of a human infection. It is also becoming clear that strain differences in the fungus and host polymorphisms can also play roles in determining the outcome of disease.

Our work in this area was supported by the Wellcome Trust and BBSRC (N.A.R.G.) and a Vidi and Vici grant of The Netherlands Organization for Scientific Research (M.G.N.).

REFERENCES

1. **Acosta-Rodriguez, E. V., L. Rivino, J. Geginat, D. Jarrossay, M. Gattorno, A. Lanzavecchia, F. Sallusto, and G. Napolitani.** 2007. Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* **8:**639–646.
2. **Agrawal, S., A. Agrawal, B. Doughty, A. Gerwitz, J. Blenis, T. van Dyke, and B. Pulendran.** 2003. Different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. *J. Immunol.* **171:**4984–4989.
3. **Akira, S., S. Uematsu, and O. Takeuchi.** 2006. Pathogen recognition and innate immunity. *Cell* **124:**783–801.
4. **Aratani, Y., H. Koyama, S. Nyui, K. Suzuki, F. Kura, and N. Maeda.** 1999. Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. *Infect. Immun.* **67:**1828–1836.
5. **Ariizumi, K., G. L. Shen, S. Shikano, R. Ritter III, P. Zukas, D. Edelbaum, A. Morita, and A. Takashima.** 2000. Cloning of a second dendritic cell-associated C-type lectin (dectin-2) and its alternatively spliced isoforms. *J. Biol. Chem.* **275:**11957–11963.
6. **Babula, O., G. Lazdane, J. Kroica, W. J. Ledger, and S. S. Witkin.** 2003. Relation between recurrent vulvovaginal candidiasis, vaginal concentrations of mannose-binding lectin, and a mannose-binding lectin gene polymorphism in Latvian women. *Clin. Infect. Dis.* **37:**733–737.
7. **Babula, O., G. Lazdane, J. Kroica, I. M. Linhares, W. J. Ledger, and S. S. Witkin.** 2005. Frequency of interleukin-4 (IL-4) -589 gene polymorphism and vaginal concentrations of IL-4, nitric oxide, and mannose-binding lectin in women with recurrent vulvovaginal candidiasis. *Clin. Infect. Dis.* **40:**1258–1262.
8. **Balish, E., R. D. Wagner, A. Vasquez-Torres, C. Pierson, and T. Warner.** 1998. Candidiasis in interferon-γ knockout (IFN-γ-/-) mice. *J. Infect. Dis.* **178:**478–487.
9. **Bellocchio, S., C. Montagnoli, S. Bozza, R. Gaziano, G. Rossi, S. S. Mambula, A. Vecchi, A. Mantovani, S. M. Levitz, and L. Romani.** 2004. The contribution of Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. *J. Immunol.* **172:**3059–3069.
10. **Ben-Ami, R., R. E. Lewis, and D. P. Kontoyiannis.** 2008. Immunocompromised hosts: immunopharmacology of modern antifungals. *Clin. Infect. Dis.* **47:**226–235.
11. **Blander, J. M., and R. Medzhitov.** 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* **440:**808–812.
12. **Blasi, E., A. Mucci, R. Neglia, F. Pezzini, B. Colombari, D. Radzioch, A. Cossarizza, E. Lugli, G. Volpini, G. Del Giudice, and S. Peppoloni.** 2005. Biological importance of the two Toll-like receptors, TLR2 and TLR4, in macrophage response to infection with *Candida albicans*. *FEMS Immunol. Med. Microbiol.* **44:**69–79.
13. **Bozza, S., T. Zelante, S. Moretti, P. Bonifazi, A. DeLuca, C. D'Angelo, G. Giovannini, C. Garlanda, L. Boon, F. Bistoni, P. Puccetti, A. Mantovani, and L. Romani.** 2008. Lack of Toll IL-1R8 exacerbates Th17 cell responses in fungal infection. *J. Immunol.* **180:**4022–4031.
14. **Brouwer, N., K. M. Dolman, M. van Houdt, M. Sta, D. Roos, and T. W. Kuijpers.** 2008. Mannose-binding lectin (MBL) facilitates opsonophagocytosis of yeasts but not of bacteria despite MBL binding. *J. Immunol.* **180:**4124–4132.
15. **Brown, G. D.** 2006. Dectin-1: a signalling non-TLR pattern-recognition receptor. *Nat. Rev. Immunol.* **6:**33–43.
16. **Brown, G. D., and S. Gordon.** 2005. Immune recognition of fungal beta-glucans. *Cell. Microbiol.* **7:**471–479.
17. **Brown, G. D., and S. Gordon.** 2001. A new receptor for beta-glucans. *Nature* **413:**36–37.
18. **Brown, G. D., J. Herre, D. L. Williams, J. A. Willment, A. S. Marshall, and S. Gordon.** 2003. Dectin-1 mediates the biological effects of beta-glucans. *J. Exp. Med.* **197:**1119–1124.
19. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J. L. Reedy, I. Agrafioti, M. B. Arnaud,**

S. Bates, A. J. Brown, S. Brunke, M. C. Costanzo, D. A. Fitzpatrick, P. W. de Groot, D. Harris, L. L. Hoyer, B. Hube, F. M. Klis, C. Kodira, N. Lennard, M. E. Logue, R. Martin, A. M. Neiman, E. Nikolaou, M. A. Quail, J. Quinn, M. C. Santos, F. F. Schmitzberger, G. Sherlock, P. Shah, K. A. Silverstein, M. S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M. P. Stumpf, P. E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N. A. Gow, M. C. Lorenz, B. W. Birren, M. Kellis, and C. A. Cuomo. 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459:**657–662.

20. **Calderone, R. A., and W. A. Fonzi.** 2001. Virulence factors of *Candida albicans*. *Trends Microbiol.* **9:**327–335.
21. **Cambi, A., K. Gijzen, J. de Vries, R. Torensma, B. Joosten, G. J. Adema, M. G. Netea, B. J. Kullberg, L. Romani, and C. G. Figdor.** 2003. The C-type lectin DC-SIGN (CD209) is an antigen-uptake receptor for *Candida albicans* on dendritic cells. *Eur. J. Immunol.* **33:**532–538.
22. **Cambi, A., M. G. Netea, H. M. Mora-Montes, N. A. Gow, S. V. Hato, D. W. Lowman, B. J. Kullberg, R. Torensma, D. L. Williams, and C. G. Figdor.** 2008. Dendritic cell interaction with *Candida albicans* critically depends on N-linked mannan. *J. Biol. Chem.* **283:**20590–20599.
23. **Casadevall, A., and L. A. Pirofski.** 2003. The damage-response framework of microbial pathogenesis. *Nat. Rev. Microbiol.* **1:**17–24.
24. **Cenci, E., A. Mencacci, G. Del Sero, C. F. d'Ostiani, P. Mosci, A. Bacci, C. Montagnoli, M. Kopf, and L. Romani.** 1998. IFN-gamma is required for IL-12 responsiveness in mice with *Candida albicans* infection. *J. Immunol.* **161:**3543–3550.
25. **Cutler, J. E.** 1991. Putative virulence factors of *Candida albicans*. *Annu. Rev. Microbiol.* **45:**187–218.
26. **Da Silva, C. A., C. Chalouni, A. Williams, D. Hartl, C. G. Lee, and J. A. Elias.** 2009. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *J. Immunol.* **182:**3573–3582.
27. **Dennehy, K. M., G. Ferwerda, I. Faro-Trindade, E. Pyz, J. A. Willment, P. R. Taylor, A. Kerrigan, S. V. Tsoni, S. Gordon, F. Meyer-Wentrup, G. J. Adema, B. J. Kullberg, E. Schweighoffer, V. Tybulewicz, H. M. Mora-Montes, N. A. Gow, D. L. Williams, M. G. Netea, and G. D. Brown.** 2008. Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur. J. Immunol.* **38:**500–506.
28. **Dillon, S., S. Agrawal, K. Banerjee, J. Letterio, T. L. Denning, K. Oswald-Richter, D. J. Kasprowicz, K. Kellar, J. Pare, T. van Dyke, S. Ziegler, D. Unutmaz, and B. Pulendran.** 2006. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J. Clin. Investig.* **116:**916–928.
29. **Eyerich, K., S. Foerster, S. Rombold, H. P. Seidl, H. Behrendt, H. Hofmann, J. Ring, and C. Traidl-Hoffmann.** 2008. Patients with chronic mucocutaneous candidiasis exhibit reduced production of Th17-associated cytokines IL-17 and IL-22. *J. Investig. Dermatol.* **128:**2640–2645.
30. **Ezekowitz, R. A. B., C. A. Sieff, M. C. Dinauer, D. G. Nathan, S. H. Orkin, and P. E. Newburger.** 1990. Restoration of phagocyte function by interferon-γ in X-linked chronic granulomatous disease occurs at the level of a progenitor cell. *Blood* **76:**2443–2448.
31. **Ferwerda, B., G. Ferwerda, T. S. Plantinga, J. A. Willment, A. B. van Spriel, H. Venselaar, C. C. Elbers, M. D. Johnson, A. Cambi, C. Huysamen, L. Jacobs, T. Jansen, K. Verheijen, L. Masthoff, S. A. Morre, G. Vriend, D. L. Williams, J. R. Perfect, L. A. Joosten, C. Wijmenga, J. W. van der Meer, G. J. Adema, B. J. Kullberg, G. D. Brown, and M. G. Netea.** 2009. Human dectin-1 deficiency and mucocutaneous fungal infections. *N. Engl. J. Med.* **361:**1760–1767.
32. **Ferwerda, G., F. Meyer-Wentrup, B. J. Kullberg, M. G. Netea, and G. J. Adema.** 2008. Dectin-1 synergizes with TLR2 and TLR4 for cytokine production in human primary monocytes and macrophages. *Cell. Microbiol.* **10:**2058–2066.
33. **Fidel, P. L., Jr.** 2007. History and update on host defense against vaginal candidiasis. *Am. J. Reprod. Immunol.* **57:**2–12.
34. **Fidel, P. L., Jr., K. A. Ginsburg, J. L. Cutright, N. A. Wolf, D. Leaman, K. Dunlap, and J. D. Sobel.** 1997. Vaginal-associated immunity in women with recurrent vulvovaginal candidiasis: evidence for vaginal Th1-type responses following intravaginal challenge with *Candida* antigen. *J. Infect. Dis.* **176:**728–739.
35. **Fradin, C., P. De Groot, D. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube.** 2005. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol. Microbiol.* **56:**397–415.
36. **Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill.** 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* **197:**1107–1117.
37. **Gantner, B. N., R. M. Simmons, and D. M. Underhill.** 2005. Dectin-1 mediates macrophage recognition of *Candida albicans* yeasts but not filaments. *EMBO J.* **24:**1277–1286.
38. **Geijtenbeek, T. B., S. J. Van Vliet, E. A. Koppel, M. Sanchez-Hernandez, C. M. Vanderbroucke-Grauls, B. Appelmelk, and Y. Van Kooyk.** 2003. Mycobacteria target DC-SIGN to suppress dendritic cell function. *J. Exp. Med.* **197:**7–17.
39. **Girardin, S. E., I. G. Boneca, L. A. M. Carneiro, A. Antignac, M. Jehanno, J. Viala, K. Tedin, M.-K. Taha, A. Labigne, U. Zahringer, A. J. Coyle, P. S. DiStefano, J. Bertin, P. J. Sansonetti, and D. J. Philpott.** 2003. Nod1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* **300:**1584–1587.
40. **Girardin, S. E., I. G. Boneca, J. Viala, M. Chamaillard, A. Labigne, G. Thomas, D. J. Philpott, and P. J. Sansonetti.** 2003. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.* **278:**8869–8872.
41. **Glocker, E. O., A. Hennigs, M. Nabavi, A. A. Schaffer, C. Woellner, U. Salzer, D. Pfeifer, H. Veelken, K. Warnatz, F. Tahami, S. Jamal, A. Manguiat, N. Rezaei, A. A. Amirzargar, A. Plebani, N. Hannesschlager, O. Gross, J. Ruland, and B. Grimbacher.** 2009. A homozygous CARD9 mutation in a family with susceptibility to fungal infections. *N. Engl. J. Med.* **361:**1727–1735.
42. **Gow, N. A. R., M. G. Netea, C. A. Munro, G. Ferwerda, S. Bates, H. M. Mora-Montes, L. Walker, T. Jansen, L. Jacobs, V. Tsoni, G. D. Brown, F. C. Odds, J. W. M. Van der Meer, A. J. P. Brown, and B. J. Kullberg.** 2007. Immune recognition of *Candida albicans* β-glucan by dectin-1. *J. Infect. Dis.* **196:**1565–1571.
43. **Gringhuis, S. I., J. den Dunnen, M. Litjens, M. van der Vlist, B. Wevers, S. C. Bruijns, and T. B. Geijtenbeek.** 2009. Dectin-1 directs T helper cell differentiation by controlling noncanonical NF-κB activation through Raf-1 and Syk. *Nat. Immunol.* **10:**203–213.
44. **Gringhuis, S. I., J. den Dunnen, M. Litjens, B. van Het Hof, Y. van Kooyk, and T. B. Geijtenbeek.** 2007. C-type lectin DC-SIGN modulates Toll-like receptor signaling via Raf-1 kinase-dependent acetylation of transcription factor NF-κB. *Immunity* **26:**605–616.

45. **Gross, O., A. Gewies, K. Finger, M. Schafer, T. Sparwasser, C. Peschel, I. Forster, and J. Ruland.** 2006. Card9 controls a non-TLR signalling pathway for innate anti-fungal immunity. *Nature* **442:**651–656.
46. **Gross, O., H. Poeck, M. Bscheider, C. Dostert, N. Hannesschlager, S. Endres, G. Hartmann, A. Tardivel, E. Schweighoffer, V. Tybulewicz, A. Mocsai, J. Tschopp, and J. Ruland.** 2009. Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence. *Nature* **459:**433–436.
47. **Hasegawa, M., R. Imamura, T. Kinoshita, N. Matsumoto, J. Masumoto, N. Inohara, and T. Suda.** 2005. ASC-mediated NF-κB activation leading to interleukin-8 production requires caspase-8 and is inhibited by CLARP. *J. Biol. Chem.* **280:**15122–15130.
48. **Heinsbroek, S. E., P. R. Taylor, F. O. Martinez, L. Martinez-Pomares, G. D. Brown, and S. Gordon.** 2008. Stage-specific sampling by pattern recognition receptors during *Candida albicans* phagocytosis. *PLoS Pathog.* **4:**e1000218.
49. **Herre, J., A. S. Marshall, E. Caron, A. D. Edwards, D. L. Williams, E. Schweighoffer, V. Tybulewicz, C. Reis e Sousa, S. Gordon, and G. D. Brown.** 2004. Dectin-1 uses novel mechanisms for yeast phagocytosis in macrophages. *Blood* **104:**4038–4045.
50. **Hirschfeld, M., J. J. Weis, V. Toshchakov, C. A. Salkowski, M. J. Cody, D. C. Ward, N. Qureshi, S. M. Michalek, and S. N. Vogel.** 2001. Signaling by Toll-like receptor 2 and 4 agonists results in differential gene expression in murine macrophages. *Infect. Immun.* **69:**1477–1482.
51. **Hise, A. G., J. Tomalka, S. Ganesan, K. Patel, B. A. Hall, G. D. Brown, and K. A. Fitzgerald.** 2009. An essential role for the NLRP3 inflammasome in host defense against the human fungal pathogen *Candida albicans*. *Cell Host Microbe* **5:**487–497.
52. **Hoebe, K., E. Janssen, and B. Beutler.** 2004. The interface between innate and adaptive immunity. *Nat. Immunol.* **5:**971–974.
53. **Hohl, T. M., M. Feldmesser, D. S. Perlin, and E. G. Pamer.** 2008. Caspofungin modulates inflammatory responses to *Aspergillus fumigatus* through stage-specific effects on fungal beta-glucan exposure. *J. Infect. Dis.* **198:**176–185.
54. **Hong, M., K. R. Ryan, P. D. Arkwright, A. R. Gennery, C. Costigan, M. Dominguez, D. W. Denning, V. McConnell, A. J. Cant, M. Abinun, G. P. Spickett, D. C. Swan, C. S. Gillespie, D. A. Young, and D. Lilic.** 2009. Pattern recognition receptor expression is not impaired in patients with chronic mucocutanous [sic] candidiasis with or without autoimmune polyendocrinopathy candidiasis ectodermal dystrophy. *Clin. Exp. Immunol.* **156:**40–51.
55. **Huang, W., L. Na, P. L. Fidel, and P. Schwarzenberger.** 2004. Requirement for interleukin-17A for systemic anti-*Candida albicans* host defense in mice. *J. Infect. Dis.* **190:**624–631.
56. **Ibata-Ombetta, S., T. Idziorek, P. A. Trinel, D. Poulain, and T. Jouault.** 2003. *Candida albicans* phospholipomannan promotes survival of phagocytosed yeasts through modulation of bad phosphorylation and macrophage apoptosis. *J. Biol. Chem.* **278:**13086–13093.
57. **Ibata-Ombetta, S., T. Idziorek, P. A. Trinel, D. Poulain, and T. Jouault.** 2003. Role of phospholipomannan in *Candida albicans* escape from macrophages and induction of cell apoptosis through regulation of bad phosphorylation. *Ann. N. Y. Acad. Sci.* **1010:**573–576.
58. **Jackson, A. P., J. A. Gamble, T. Yeomans, G. P. Moran, D. Saunders, D. Harris, M. Aslett, J. F. Barrell, G. Butler, F. Citiulo, D. C. Coleman, P. W. de Groot, T. J. Goodwin, M. A. Quail, J. McQuillan, C. A. Munro, A. Pain, R. T. Poulter, M. A. Rajandream, H. Renauld, M. J. Spiering, A. Tivey, N. A. Gow, B. Barrell, D. J. Sullivan, and M. Berriman.** 2009. Comparative genomics of the fungal pathogens *Candida dubliniensis* and *Candida albicans*. *Genome Res.* **19:**2231–2244.
59. **Jawhara, S., X. Thuru, A. Standaert-Vitse, T. Jouault, S. Mordon, B. Sendid, P. Desreumaux, and D. Poulain.** 2008. Colonization of mice by *Candida albicans* is promoted by chemically induced colitis and augments inflammatory responses through galectin-3. *J. Infect. Dis.* **197:**972–980.
60. **Joly, S., N. Ma, J. J. Sadler, D. R. Soll, S. L. Cassel, and F. S. Sutterwala.** 2009. Cutting edge: *Candida albicans* hyphae formation triggers activation of the Nlrp3 inflammasome. *J. Immunol.* **183:**3578–3581.
61. **Jouault, T., M. El Abed-El Behi, M. Martinez-Esparza, L. Breuilh, P. A. Trinel, M. Chamaillard, F. Trottein, and D. Poulain.** 2006. Specific recognition of *Candida albicans* by macrophages requires galectin-3 to discriminate *Saccharomyces cerevisiae* and needs association with TLR2 for signaling. *J. Immunol.* **177:**4679–4687.
62. **Jouault, T., S. Ibata-Ombetta, O. Takeuchi, P.-A. Trinel, P. Sacchetti, P. Lefebvre, S. Akira, and D. Poulain.** 2003. *Candida albicans* phospholipomannan is sensed through Toll-like receptors. *J. Infect. Dis.* **188:**165–172.
63. **Kaposzta, R., P. Tree, L. Marodi, and S. Gordon.** 1998. Characteristics of invasive candidiasis in gamma interferon- and interleukin-4-deficient mice: role of macrophages in host defense against *Candida albicans*. *Infect. Immun.* **66:**1708–1717.
64. **Kennedy, A. D., J. A. Willment, D. W. Dorward, D. L. Williams, G. D. Brown, and F. R. DeLeo.** 2007. Dectin-1 promotes fungicidal activity of human neutrophils. *Eur. J. Immunol.* **37:**467–478.
65. **Kilpatrick, D. C.** 2002. Mannan-binding lectin: clinical significance and applications. *Biochim. Biophys. Acta* **1572:**401–413.
66. **Klis, F. M., P. de Groot, and K. Hellingwerf.** 2001. Molecular organization of the cell wall of *Candida albicans*. *Med. Mycol.* **39**(Suppl. 1)**:**1–8.
67. **Klis, F. M., G. J. Sosinska, P. W. de Groot, and S. Brul.** 2009. Covalently linked cell wall proteins of *Candida albicans* and their role in fitness and virulence. *FEMS Yeast Res.* **9:**1013–1028.
68. **Kohatsu, L., D. K. Hsu, A. G. Jegalian, F. T. Liu, and L. G. Baum.** 2006. Galectin-3 induces death of *Candida* species expressing specific beta-1,2-linked mannans. *J. Immunol.* **177:**4718–4726.
69. **Koppel, E. A., K. P. van Gisbergen, T. B. Geijtenbeek, and Y. van Kooyk.** 2005. Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. *Cell. Microbiol.* **7:**157–165.
70. **Koyama, T., M. Makita, N. Shibata, and Y. Okawa.** 2009. Influence of oxidative and osmotic stresses on the structure of the cell wall mannan of *Candida albicans* serotype A. *Carbohydr. Res.* **344:**2195–2200.
71. **Kozel, T. R.** 1996. Activation of the complement system by pathogenic fungi. *Clin. Microbiol. Rev.* **9:**34–46.
72. **Kozel, T. R., L. C. Weinhold, and D. M. Lupan.** 1996. Distinct characteristics of initiation of the classical and alternative complement pathways by *Candida albicans*. *Infect. Immun.* **64:**3360–3368.
73. **Kumar, H., Y. Kumagai, T. Tsuchida, P. A. Koenig, T. Satoh, Z. Guo, M. H. Jang, T. Saitoh, S. Akira, and T. Kawai.** 2009. Involvement of the NLRP3 inflammasome

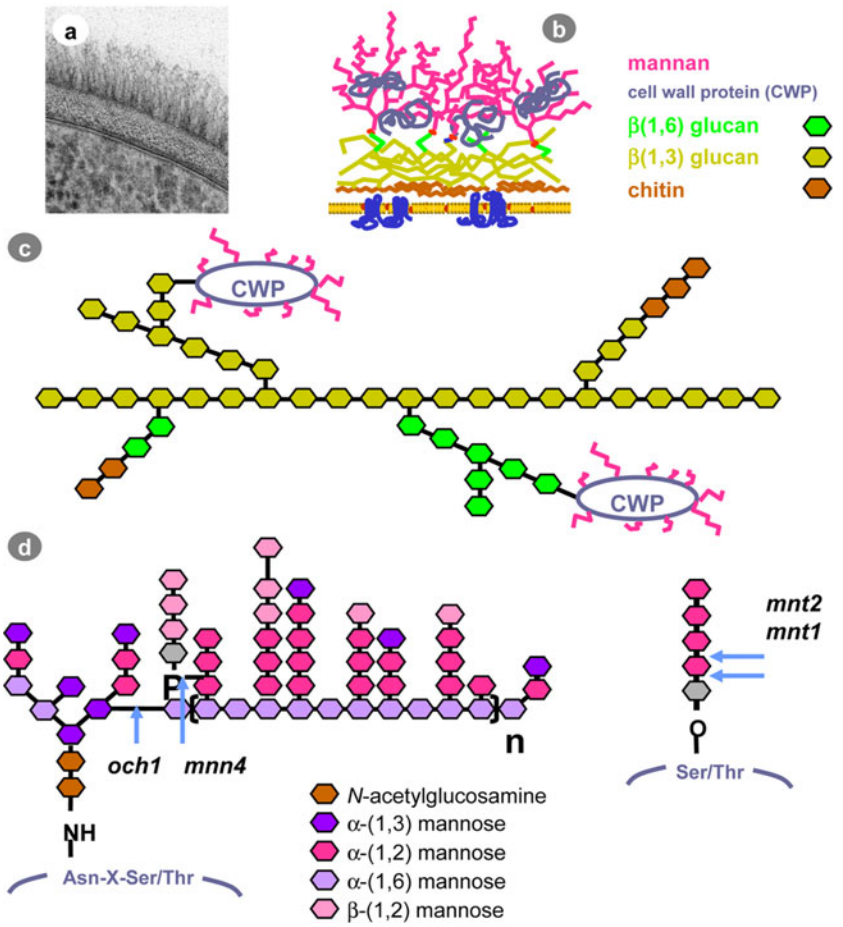
in innate and humoral adaptive immune responses to fungal beta-glucan. *J. Immunol.* **183:**8061–8067.
74. **Lamaris, G. A., R. E. Lewis, G. Chamilos, G. S. May, A. Safdar, T. J. Walsh, I. I. Raad, and D. P. Kontoyiannis.** 2008. Caspofungin-mediated beta-glucan unmasking and enhancement of human polymorphonuclear neutrophil activity against *Aspergillus* and non-*Aspergillus* hyphae. *J. Infect. Dis.* **198:**186–192.
75. **Lavigne, L. M., L. R. Schopf, C. L. Chung, R. Maylor, and J. P. Sypek.** 1998. The role of recombinant IL-12 and IFN-gamma in the pathogenesis of a murine *Candida albicans* infection. *J. Immunol.* **160:**284–292.
76. **Le Cabec, V., L. J. Emorine, I. Toesca, C. Cougoule, and I. Maridonneau-Parini.** 2005. The human macrophage mannose receptor is not a professional phagocytic receptor. *J. Leukoc. Biol.* **77:**934–943.
77. **Lee, C. G., C. A. Da Silva, J. Y. Lee, D. Hartl, and J. A. Elias.** 2008. Chitin regulation of immune responses: an old molecule with new roles. *Curr. Opin. Immunol.* **20:**684–689.
78. **Lee, S. J., N. Y. Zheng, M. Clavijo, and M. C. Nussenzweig.** 2003. Normal host defense during systemic candidiasis in mannose receptor-deficient mice. *Infect. Immun.* **71:**437–445.
79. **Leibundgut-Landmann, S., O. Gross, M. J. Robinson, F. Osorio, E. C. Slack, S. V. Tsoni, E. Schweighoffer, V. Tybulewicz, G. D. Brown, J. Ruland, and E. S. C. Reis.** 2007. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat. Immunol.* **8:**630–638.
80. **Lemaitre, B., E. Nicolas, L. Michaut, J.-M. Reichhart, and J. A. Hoffmann.** 1996. The dorsoventral regulatory gene cassette Spaetzle/Toll/Cactus controls the potent antifungal response in *Drosophila* adults. *Cell* **86:**973–983.
81. **Lev-Sagie, A., D. Prus, I. M. Linhares, Y. Lavy, W. J. Ledger, and S. S. Witkin.** 2009. Polymorphism in a gene coding for the inflammasome component NALP3 and recurrent vulvovaginal candidiasis in women with vulvar vestibulitis syndrome. *Am. J. Obstet. Gynecol.* **200:** 303.e1–303.e6.
82. **Li, M., Q. Chen, Y. Shen, and W. Liu.** 2009. *Candida albicans* phospholipomannan triggers inflammatory responses of human keratinocytes through Toll-like receptor 2. *Exp. Dermatol.* **18:**603–610.
83. **Lipscombe, R. J., M. Sumiya, J. A. Summerfield, and M. W. Turner.** 1995. Distinct physicochemical characteristics of human mannose binding protein expressed by individuals of differing genotype. *Immunology* **85:** 660–667.
84. **Lo, H.-J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90:**939–949.
85. **Malmvall, B. E., and P. Follin.** 1993. Successful interferon-gamma therapy in a chronic granulomatous disease (CGD) patient suffering from *Staphylococcus aureus* hepatic abscess and invasive *Candida albicans* infection. *Scand. J. Infect. Dis.* **25:**61–66.
86. **Mansour, M. K., and S. M. Levitz.** 2002. Interactions of fungi with phagocytes. *Curr. Opin. Microbiol.* **5:**359–365.
87. **Marodi, L., S. Schreiber, D. C. Anderson, R. P. MacDermott, H. M. Korchak, and R. B. Johnston, Jr.** 1993. Enhancement of macrophage candidacidal activity by interferon-γ. Increased phagocytosis, killing, and calcium signal mediated by a decreased number of mannose receptors. *J. Clin. Investig.* **91:**2596–2601.
88. **Martin, B., K. Hirota, D. J. Cua, B. Stockinger, and M. Veldhoen.** 2009. Interleukin-17-producing γδ T cells selectively expand in response to pathogen products and environmental signals. *Immunity* **31:**321–330.
89. **Martinon, F., and J. Tschopp.** 2004. Inflammatory caspases: linking an intracellular innate immune system to autoinflammatory diseases. *Cell* **117:**561–574.
90. **McGreal, E. P., M. Rosas, G. D. Brown, S. Zamze, S. Y. Wong, S. Gordon, L. Martinez-Pomares, and P. R. Taylor.** 2006. The carbohydrate-recognition domain of Dectin-2 is a C-type lectin with specificity for high mannose. *Glycobiology* **16:**422–430.
91. **McKenzie, C. G., U. Koser, L. E. Lewis, J. M. Bain, H. M. Mora-Montes, R. N. Barker, N. A. Gow, and L. P. Erwig.** 2010. Contribution of *Candida albicans* cell wall components to recognition by and escape from murine macrophages. *Infect. Immun.* **78:**1650–1658.
92. **Means, T. K., E. Mylonakis, E. Tampakakis, R. A. Colvin, E. Seung, L. Puckett, M. F. Tai, C. R. Stewart, R. Pukkila-Worley, S. E. Hickman, K. J. Moore, S. B. Calderwood, N. Hacohen, A. D. Luster, and J. El Khoury.** 2009. Evolutionarily conserved recognition and innate immunity to fungal pathogens by the scavenger receptors SCARF1 and CD36. *J. Exp. Med.* **206:** 637–653.
93. **Medzhitov, R.** 2001. Toll-like receptors and innate immunity. *Nat. Rev. Immunol.* **1:**135–145.
94. **Meisel, C., J. C. Schefold, R. Pschowski, T. Baumann, K. Hetzger, J. Gregor, S. Weber-Carstens, D. Hasper, D. Keh, H. Zuckermann, P. Reinke, and H. D. Volk.** 2009. Granulocyte-macrophage colony-stimulating factor to reverse sepsis-associated immunosuppression: a double-blind, randomized, placebo-controlled multicenter trial. *Am. J. Respir. Crit. Care Med.* **180:**640–648.
95. **Mencacci, A., A. Bacci, E. Cenci, C. Montagnoli, S. Fiorucci, A. Casagrande, R. A. Flavell, F. Bistoni, and L. Romani.** 2000. Interleukin 18 restores defective Th1 immunity to *Candida albicans* in caspase 1-deficient mice. *Infect. Immun.* **68:**5126–5131.
96. **Milner, J. D., J. M. Brenchley, A. Laurence, A. F. Freeman, B. J. Hill, K. M. Elias, Y. Kanno, C. Spalding, H. Z. Elloumi, M. L. Paulson, J. Davis, A. Hsu, A. I. Asher, J. O'Shea, S. M. Holland, W. E. Paul, and D. C. Douek.** 2008. Impaired T_H17 cell differentiation in subjects with autosomal dominant hyper-IgE syndrome. *Nature* **452:**773–776.
97. **Mitchison, A.** 1993. Will we survive? *Sci. Am.* **269:**136–144.
98. **Miyazato, A., K. Nakamura, N. Yamamoto, H. M. Mora-Montes, M. Tanaka, Y. Abe, D. Tanno, K. Inden, X. Gang, K. Ishii, K. Takeda, S. Akira, S. Saijo, Y. Iwakura, Y. Adachi, N. Ohno, K. Mitsutake, N. A. Gow, M. Kaku, and K. Kawakami.** 2009. Toll-like receptor 9-dependent activation of myeloid dendritic cells by deoxynucleic acids from *Candida albicans*. *Infect. Immun.* **77:**3056–3064.
99. **Morre, S. A., L. S. Murillo, J. Spaargaren, H. S. Fennema, and A. S. Pena.** 2002. Role of Toll-like receptor 4 Asp299Gly polymorphism in susceptibility to *Candida albicans* infection. *J. Infect. Dis.* **186:**1377–1379.
100. **Munro, C. A., S. Bates, E. T. Buurman, H. B. Hughes, D. M. Maccallum, G. Bertram, A. Atrih, M. A. Ferguson, J. M. Bain, A. Brand, S. Hamilton, C. Westwater, L. M. Thomson, A. J. Brown, F. C. Odds, and N. A. Gow.** 2005. Mnt1p and Mnt2p of *Candida albicans* are partially redundant alpha-1,2-mannosyltransferases that participate in O-linked mannosylation and are required for adhesion and virulence. *J. Biol. Chem.* **280:** 1051–1060.
101. **Murciano, C., E. Villamon, D. Gozalbo, P. Roig, J. E. O'Connor, and M. L. Gil.** 2006. Toll-like receptor 4 defective mice carrying point or null mutations do not show increased susceptibility to *Candida albicans* in a

model of hematogenously disseminated infection. *Med. Mycol.* **44:**149–157.

102. **Murciano, C., A. Yanez, M. L. Gil, and D. Gozalbo.** 2007. Both viable and killed *Candida albicans* cells induce in vitro production of TNF-alpha and IFN-gamma in murine cells through a TLR2-dependent signalling. *Eur. Cytokine Netw.* **18:**38–43.
103. **Netea, M. G., G. D. Brown, B. J. Kullberg, and N. A. Gow.** 2008. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat. Rev. Microbiol.* **6:**67–78.
104. **Netea, M. G., C. de Graaf, A. Vonk, I. Verschueren, J. W. M. Van der Meer, and B. J. Kullberg.** 2002. The role of Toll-like receptors in the defense against disseminated candidiasis. *J. Infect. Dis.* **185:**1483–1489.
105. **Netea, M. G., N. A. Gow, L. A. Joosten, I. Verschueren, J. W. van der Meer, and B. J. Kullberg.** 2010. Variable recognition of *Candida albicans* strains by TLR4 and lectin recognition receptors. *Med. Mycol.* **48:**897–903.
106. **Netea, M. G., N. A. Gow, C. A. Munro, S. Bates, C. Collins, G. Ferwerda, R. P. Hobson, G. Bertram, H. B. Hughes, T. Jansen, L. Jacobs, E. T. Buurman, K. Gijzen, D. L. Williams, R. Torensma, A. McKinnon, D. M. MacCallum, F. C. Odds, J. W. Van der Meer, A. J. Brown, and B. J. Kullberg.** 2006. Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Investig.* **116:**1642–1650.
107. **Netea, M. G., C. A. Nold-Petry, M. F. Nold, L. A. Joosten, B. Opitz, J. H. van der Meer, F. L. van de Veerdonk, G. Ferwerda, B. Heinhuis, I. Devesa, C. J. Funk, R. J. Mason, B. J. Kullberg, A. Rubartelli, J. W. van der Meer, and C. A. Dinarello.** 2009. Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages. *Blood* **113:**2324–2335.
108. **Netea, M. G., R. Sutmuller, C. Hermann, C. A. A. Van der Graaf, J. W. M. Van der Meer, G. Adema, and B. J. Kullberg.** 2004. Toll-like receptor 2 inhibits cellular responses against *Candida albicans* through pathways mediated by IL-10 and CD4+CD25+ regulatory T cells. *J. Immunol.* **172:**3712–3718.
109. **Netea, M. G., F. van de Veerdonk, I. Verschueren, J. W. van der Meer, and B. J. Kullberg.** 2008. Role of TLR1 and TLR6 in the host defense against disseminated candidiasis. *FEMS Immunol. Med. Microbiol.* **52:**118–123.
110. **Netea, M. G., C. Van der Graaf, J. W. M. Van der Meer, and B. J. Kullberg.** 2004. The role of Toll-like receptors in host defense: bringing specificity to the innate immune response. *J. Leukoc. Biol.* **75:**749–755.
111. **O'Neill, L. A.** 2002. Signal transduction pathways activated by the IL-1 receptor/Toll-like receptor superfamily. *Curr. Top. Microbiol. Immunol.* **270:**47–61.
112. **Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem.** 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. USA* **97:**13766–13771.
113. **Plantinga, T. S., W. J. van der Velden, B. Ferwerda, A. B. van Spriel, G. Adema, T. Feuth, J. P. Donnelly, G. D. Brown, B. J. Kullberg, N. M. Blijlevens, and M. G. Netea.** 2009. Early stop polymorphism in human DECTIN-1 is associated with increased *Candida* colonization in hematopoietic stem cell transplant recipients. *Clin. Infect. Dis.* **49:**724–732.
114. **Poltorak, A., X. He, I. Smirnova, M.-Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton, and B. Beutler.** 1998. Defective LPS signaling in C3H/HeJ and C57Bl/10ScCr mice: mutations in TLR4 gene. *Science* **282:**2085–2088.
115. **Reese, T. A., H. E. Liang, A. M. Tager, A. D. Luster, N. Van Rooijen, D. Voehringer, and R. M. Locksley.** 2007. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* **447:**92–96.
116. **Reeves, E. P., H. Lu, H. L. Jacobs, C. G. Messina, S. Bolsover, G. Gabella, E. O. Potma, A. Warley, J. Roes, and A. W. Segal.** 2002. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature* **416:**291–297.
117. **Robinson, M. J., F. Osorio, M. Rosas, R. P. Freitas, E. Schweighoffer, O. Gross, J. S. Verbeek, J. Ruland, V. Tybulewicz, G. D. Brown, L. F. Moita, P. R. Taylor, and C. Reis e Sousa.** 2009. Dectin-2 is a Syk-coupled pattern recognition receptor crucial for Th17 responses to fungal infection. *J. Exp. Med.* **206:**2037–2051.
118. **Rock, F. L., G. Hardiman, J. C. Timans, J. A. Kastelein, and J. F. Bazan.** 1998. A family of human receptors structurally related to *Drosophila* Toll. *Proc. Natl. Acad. Sci. USA* **95:**588–593.
119. **Rogers, N. C., E. C. Slack, A. D. Edwards, M. A. Nolte, O. Schulz, E. Schweighoffer, D. L. Williams, S. Gordon, V. L. Tybulewicz, G. D. Brown, and C. Reis e Sousa.** 2005. Syk-dependent cytokine induction by Dectin-1 reveals a novel pattern recognition pathway for C type lectins. *Immunity* **22:**507–517.
120. **Romani, L.** 2004. Immunity to fungal infections. *Nat. Rev. Immunol.* **4:**1–13.
121. **Romani, L., C. Montagnoli, S. Bozza, K. Perruccio, A. Spreca, P. Allavena, S. Verbeek, R. A. Calderone, F. Bistoni, and P. Puccetti.** 2004. The exploitation of distinct recognition receptors in dendritic cells determines the full range of host immune relationships with *Candida albicans*. *Int. Immunol.* **16:**149–161.
122. **Rubin-Bejerano, I., C. Abeijon, P. Magnelli, P. Grisafi, and G. R. Fink.** 2007. Phagocytosis by human neutrophils is stimulated by a unique fungal cell wall component. *Cell Host Microbe* **2:**55–67.
123. **Saegusa, S., M. Totsuka, S. Kaminogawa, and T. Hosoi.** 2004. *Candida albicans* and *Saccharomyces cerevisiae* induce interleukin-8 production from intestinal epithelial-like Caco-2 cells in the presence of butyric acid. *FEMS Immunol. Med. Microbiol.* **41:**227–235.
124. **Safdar, A., G. H. Rodriguez, B. Lichtiger, B. F. Dickey, D. P. Kontoyiannis, E. J. Freireich, E. J. Shpall, I. I. Raad, H. M. Kantarjian, and R. E. Champlin.** 2006. Recombinant interferon γ1b immune enhancement in 20 patients with hematologic malignancies and systemic opportunistic infections treated with donor granulocyte transfusions. *Cancer* **106:**2664–2671.
125. **Saijo, S., N. Fujikado, T. Furuta, S. H. Chung, H. Kotaki, K. Seki, K. Sudo, S. Akira, Y. Adachi, N. Ohno, T. Kinjo, K. Nakamura, K. Kawakami, and Y. Iwakura.** 2007. Dectin-1 is required for host defense against *Pneumocystis carinii* but not against *Candida albicans*. *Nat. Immunol.* **8:**39–46.
126. **Sato, K., X. L. Yang, T. Yudate, J. S. Chung, J. Wu, K. Luby-Phelps, R. P. Kimberly, D. Underhill, P. D. Cruz, Jr., and K. Ariizumi.** 2006. Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *J. Biol. Chem.* **281:**38854–38866.
127. **Saulsbury, F. T.** 2001. Successful treatment of *Aspergillus* brain abscess with itraconazole and interferon-γ in a patient with chronic granulomatous disease. *Clin. Infect. Dis.* **32:**E137–E139.

128. **Savolainen, J., A. Rantala, M. Nermes, L. Lehtonen, and M. Viander.** 1996. Enhanced IgE response to *Candida albicans* in postoperative invasive candidiasis. *Clin. Exp. Allergy* **26:**452–460.
129. **Stahl, P. D., and R. A. Ezekowitz.** 1998. The mannose receptor is a pattern recognition receptor involved in host defense. *Curr. Opin. Immunol.* **10:**50–55.
130. **Stahl, P. D., J. S. Rodman, M. J. Miller, and P. H. Schlesinger.** 1978. Evidence for receptor-mediated binding of glycoproteins, glycoconjugates, and lysosomal glycosidases by alveolar macrophages. *Proc. Natl. Acad. Sci. USA* **75:**1399–1403.
131. **Stevens, D. A., B. J. Kullberg, E. Brummer, A. Casadevall, M. G. Netea, and A. M. Sugar.** 2000. Combined treatment: antifungal drugs with antibodies, cytokines or drugs. *Med. Mycol.* **38**(Suppl. 1)**:**305–315.
132. **Stuyt, R. J., M. G. Netea, I. Verschueren, G. Fantuzzi, C. A. Dinarello, J. W. M. Van der Meer, and B. J. Kullberg.** 2002. Role of interleukin-18 in host defense against disseminated *Candida albicans* infection. *Infect. Immun.* **70:**3284–3286.
133. **Sutmuller, R. P., M. H. den Brok, M. Kramer, E. J. Bennink, L. W. Toonen, B. J. Kullberg, L. A. Joosten, S. Akira, M. G. Netea, and G. J. Adema.** 2006. Toll-like receptor 2 controls expansion and function of regulatory T cells. *J. Clin. Investig.* **116:**485–494.
134. **Tada, H., E. Nemoto, H. Shimauki, T. Watanabe, T. Mikami, T. Matsumoto, N. Ohna, H. Tamura, K. Shibata, S. Akashi, K. Miyake, S. Sugawara, and H. Takada.** 2002. *Saccharomyces cerevisiae*- and *Candida albicans*-derived mannan induced production of tumor necrosis factor alpha by human monocytes in a CD14- and Toll-like receptor 4-dependent manner. *Microbiol. Immunol.* **46:**503–512.
135. **Taylor, P. R., S. V. Tsoni, J. A. Willment, K. M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, and G. D. Brown.** 2007. Dectin-1 is required for beta-glucan recognition and control of fungal infection. *Nat. Immunol.* **8:**31–38.
136. **Toshchakov, V., B. W. Jones, P.-Y. Perera, K. Thomas, M. Joshua Cody, S. Zhang, B. R. G. Williams, J. Major, T. A. Hamilton, M. J. Fenton, and S. N. Vogel.** 2002. TLR4, but not TLR2, mediates IFNβ-induced STAT I α/β-dependent gene expression in macrophages. *Nat. Immunol.* **3:**392–398.
137. **van Bruggen, R., A. Drewniak, M. Jansen, M. van Houdt, D. Roos, H. Chapel, A. J. Verhoeven, and T. W. Kuijpers.** 2009. Complement receptor 3, not Dectin-1, is the major receptor on human neutrophils for beta-glucan-bearing particles. *Mol. Immunol.* **47:**575–581.
138. **van der Graaf, C. A., M. G. Netea, B. Franke, S. E. Girardin, J. W. van der Meer, and B. J. Kullberg.** 2006. Nucleotide oligomerization domain 2 (Nod2) is not involved in the pattern recognition of *Candida albicans*. *Clin. Vaccine Immunol.* **13:**423–425.
139. **Van der Graaf, C. A., M. G. Netea, S. A. Morre, M. Den Heijer, P. E. Verweij, J. W. Van der Meer, and B. J. Kullberg.** 2006. Toll-like receptor 4 Asp299Gly/Thr399Ile polymorphisms are a risk factor for *Candida* bloodstream infection. *Eur. Cytokine Netw.* **17:**29–34.
140. **van der Graaf, C. A. A., M. G. Netea, J. P. H. Drenth, R. H. te Morsche, J. W. M. Van der Meer, and B. J. Kullberg.** 2003. *Candida*-specific interferon-gamma deficiency and Toll-like receptor polymorphisms in patients with chronic mucocutaneous candidiasis. *Neth. J. Med.* **61:**365–369.
141. **van der Graaf, C. A. A., M. G. Netea, I. Verschueren, J. W. M. van der Meer, and B. J. Kullberg.** 2005. Differential cytokine production and Toll-like receptor signaling pathways by *Candida albicans* blastoconidia and hyphae. *Infect. Immun.* **73:**7458–7464.
142. **van de Veerdonk, F. L., B. J. Kullberg, J. W. van der Meer, N. A. Gow, and M. G. Netea.** 2008. Host-microbe interactions: innate pattern recognition of fungal pathogens. *Curr. Opin. Microbiol.* **11:**305–312.
143. **van de Veerdonk, F. L., R. Marijnissen, L. A. Joosten, B. J. Kullberg, J. P. Drenth, M. G. Netea, and J. W. van der Meer.** 2009. Milder clinical hyperimmunoglobulin E syndrome phenotype is associated with partial interleukin-17 deficiency. *Clin. Exp. Immunol.* **159:**57–64.
144. **van de Veerdonk, F. L., R. J. Marijnissen, B. J. Kullberg, H. J. Koenen, S. C. Cheng, I. Joosten, W. B. van den Berg, D. L. Williams, J. W. van der Meer, L. A. Joosten, and M. G. Netea.** 2009. The macrophage mannose receptor induces IL-17 in response to *Candida albicans*. *Cell Host Microbe* **5:**329–340.
145. **van de Veerdonk, F. L., M. G. Netea, T. J. Jansen, L. Jacobs, I. Verschueren, J. W. van der Meer, and B. J. Kullberg.** 2008. Redundant role of TLR9 for anti-*Candida* host defense. *Immunobiology* **213:**613–620.
146. **van Spriel, A. B., M. Sofi, K. H. Gartlan, A. van der Schaaf, I. Verschueren, R. Torensma, R. A. Raymakers, B. E. Loveland, M. G. Netea, G. J. Adema, M. D. Wright, and C. G. Figdor.** 2009. The tetraspanin protein CD37 regulates IgA responses and anti-fungal immunity. *PLoS Pathog.* **5:**e1000338.
147. **Vignali, D. A., L. W. Collison, and C. J. Workman.** 2008. How regulatory T cells work. *Nat. Rev. Immunol.* **8:**523–532.
148. **Villamon, E., D. Gozalbo, P. Roig, J. E. O'Connor, D. Fradelizi, and M. L. Gil.** 2004. Toll-like receptor-2 is essential in murine defenses against *Candida albicans* infections. *Microbes Infect.* **6:**1–7.
149. **Villiers, C., M. Chevallet, H. Diemer, R. Couderc, H. Freitas, A. Van Dorsselaer, P. N. Marche, and T. Rabilloud.** 2009. From secretome analysis to immunology: chitosan induces major alterations in the activation of dendritic cells via a TLR4-dependent mechanism. *Mol. Cell. Proteomics* **8:**1252–1264.
150. **Vonk, A. G., M. G. Netea, J. H. van Krieken, Y. Iwakura, J. W. van der Meer, and B. J. Kullberg.** 2006. Endogenous interleukin (IL)-1 alpha and IL-1 beta are crucial for host defense against disseminated candidiasis. *J. Infect. Dis.* **193:**1419–1426.
151. **Walker, L. A., C. A. Munro, I. de Bruijn, M. D. Lenardon, A. McKinnon, and N. A. Gow.** 2008. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. *PLoS Pathog.* **4:**e1000040.
152. **Weindl, G., J. R. Naglik, S. Kaesler, T. Biedermann, B. Hube, H. C. Korting, and M. Schaller.** 2007. Human epithelial cells establish direct antifungal defense through TLR4-mediated signaling. *J. Clin. Investig.* **117:**3664–3672.
153. **Wells, C. A., J. A. Salvage-Jones, X. Li, K. Hitchens, S. Butcher, R. Z. Murray, A. G. Beckhouse, Y. L. Lo, S. Manzanero, C. Cobbold, K. Schroder, B. Ma, S. Orr, L. Stewart, D. Lebus, P. Sobieszczuk, D. A. Hume, J. Stow, H. Blanchard, and R. B. Ashman.** 2008. The macrophage-inducible C-type lectin, mincle, is an essential component of the innate immune response to *Candida albicans*. *J. Immunol.* **180:**7404–7413.
154. **Wheeler, R. T., and G. R. Fink.** 2006. A drug-sensitive genetic network masks fungi from the immune system. *PLoS Pathog.* **2:**328–339.
155. **Wheeler, R. T., D. Kombe, S. D. Agarwala, and G. R. Fink.** 2008. Dynamic, morphotype-specific *Candida albicans* beta-glucan exposure during infection and drug treatment. *PLoS Pathog.* **4:**e1000227.

156. **Witkin, S. S., S. Gerber, and W. J. Ledger.** 2002. Differential characterization of women with vulvar vestibulitis syndrome. *Am. J. Obstet. Gynecol.* **187:**589–594.
157. **Woehrle, T., W. Du, A. Goetz, H. Y. Hsu, T. O. Joos, M. Weiss, U. Bauer, U. B. Brueckner, and E. Marion Schneider.** 2008. Pathogen specific cytokine release reveals an effect of TLR2 Arg753Gln during *Candida* sepsis in humans. *Cytokine* **41:**322–329.
158. **Wozniok, I., A. Hornbach, C. Schmitt, M. Frosch, H. Einsele, B. Hube, J. Loffler, and O. Kurzai.** 2008. Induction of ERK-kinase signalling triggers morphotype-specific killing of *Candida albicans* filaments by human neutrophils. *Cell. Microbiol.* **10:**807–820.
159. **Yoon, J., J. U. Ponikau, C. B. Lawrence, and H. Kita.** 2008. Innate antifungal immunity of human eosinophils mediated by a beta 2 integrin, CD11b. *J. Immunol.* **181:**2907–2915.
160. **Zelante, T., A. De Luca, P. Bonifazi, C. Montagnoli, S. Bozza, S. Moretti, M. L. Belladonna, C. Vacca, C. Conte, P. Mosci, F. Bistoni, P. Puccetti, R. A. Kastelein, M. Kopf, and L. Romani.** 2007. IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance. *Eur. J. Immunol.* **37:** 2695–2706.
161. **Zelensky, A. N., and J. E. Gready.** 2005. The C-type lectin-like domain superfamily. *FEBS J.* **272:** 6179–6217.
162. **Zhou, M., B. Yang, R. Ma, and C. Wu.** 2008. Memory Th-17 cells specific for *C. albicans* are persistent in human peripheral blood. *Immunol. Lett.* **118:**72–81.



COLOR PLATE 3 (CHAPTER 11) Overview of the structure of *Candida* cell wall and its main PAMPs. (a) Freeze-substitution electron micrograph showing the outer fibrillar mannoprotein layer and the inner amorphous skeletal layer. (b to d) Representations of the arrangement and structures of components of the *C. albicans* cell wall. In panel c the linkages of cell wall proteins to the skeletal β-1,3 glucan–chitin layer are shown, and in panel d the structure of the *O*-linked and *N*-linked mannans that are attached to serine/threonine and asparagine residues (respectively) of glycosylated proteins is described. 10.1128/9781555817176.ch11cp3

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch12

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Vaccines and Passive Immunity against Candidiasis

BRAD SPELLBERG, YUE FU, AND ASHRAF S. IBRAHIM

INTRODUCTION

The second half of the 20th century witnessed revolutionary advances in the treatment of critically ill patients. Such advances have helped save countless lives, but as a side effect, each has also contributed to the rise of a new type of nosocomial infection which has increasingly affected hospitals in countries with advanced medical technology: invasive fungal infections (119). *Candida* is the most common cause of invasive fungal infections in the United States and in other countries with advanced medical technology (119). *Candida* spp. are now statistically tied with *Enterococcus* as the third most frequent nosocomial bloodstream isolates (161–163), surpassing the incidence of bacteremia caused by *Escherichia coli* or *Klebsiella* species. Indeed, *Candida* accounts for up to 10% of all nosocomial bloodstream infections and 11% of all catheter-related infections (4). The cost associated with hematogenously disseminated candidiasis is $2 billion to $4 billion/year in the United States (95, 159, 167). Even with antifungal therapy, disseminated candidiasis has an unacceptable attributable mortality rate of 30 to 40% (1, 43, 110), and a >50% attributable mortality rate in myeloablated patients (88, 104, 158). The mortality rate of *Candida* sepsis is also greater than 50% and is therefore higher than the rate of mortality from sepsis due to *Pseudomonas aeruginosa*, *Staphylococcus aureus*, or *E. coli* (73, 106). Furthermore, resistance to conventional antifungal therapies among *Candida* isolates is rising (18, 62, 87, 96, 155). Hence, new strategies to prevent and treat candidal infections are obviously needed.

A vaccine to prevent candidiasis is particularly attractive because clinical risk factors for the disease are well defined and these risk factors are increasingly common in the United States and worldwide (109, 113). The ability to prospectively identify patients at risk for candidiasis will facilitate both clinical trials and, ultimately, application of a vaccine. Passive immunotherapy as an adjunct to antifungal therapy in patients with established invasive candidal infections is also of promise to improve the outcomes of such infections. We review the rationale for and latest advances in development of passive and active immunization against invasive candidal infections.

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RATIONALE FOR IMMUNOPROPHYLACTIC AND THERAPEUTIC STRATEGIES FOR INVASIVE CANDIDAL INFECTIONS

Epidemiology of Disseminated Candidiasis

Population-based surveys of candidemia in major metropolitan areas in the United States have reported that the annual incidence of disseminated candidiasis is approximately 20 cases per 100,000 population (45, 59, 159). In high-risk/hospitalized patients, this incidence increases up to 50-fold (59, 82, 102, 119, 123). In recent observational studies, 0.5 to 1% of all patients admitted to medical or surgical services (36, 119, 123) or neonatal intensive care units (69, 128) had candidemia. These incidences represented 15- to 20-fold increases compared to a decade earlier (36, 69). Similar temporal increases in the incidence of candidemia have been noted in other studies (61, 71, 115, 125). In summary, the increasingly common target populations for active immunization against disseminated candidiasis include patients who are in intensive care units (including premature neonates), are receiving broad-spectrum antibiotics, have burn injuries, have central venous catheters, have undergone elective or emergent intra-abdominal or cardiac surgery, or have received parenteral nutrition. These groups comprise an enormous population of patients (in the millions every year) in the United States who are acutely at risk for disseminated candidiasis.

Only a minority (i.e., <20%) of patients at risk for disseminated candidiasis have profound defects in host immune function. The major form of immunosuppression that predisposes to development of disseminated candidiasis is a defect in innate phagocytic activity. Neutropenia dramatically increases the risk of (22, 54, 60, 88, 94, 104) and mortality due to (2, 88, 104, 108, 153, 154) disseminated candidiasis. Concordant with their well-characterized suppression of

phagocyte function (52, 148, 168), glucocorticoids also increase the risk of disseminated candidiasis (51, 104, 154). Patients with late-stage human immunodeficiency virus (HIV) disease have an extremely high incidence of developing mucocutaneous candidiasis (28, 117). While patients infected with HIV also develop invasive candidal infections at a high rate, this increased incidence of candidemia is attributable to the increased incidence of the usual risk factors for candidemia, including central lines, broad-spectrum antibiotics, hospitalization in an intensive care unit, parenteral nutrition, and neutropenia (74, 152). HIV patients without these typical risk factors are not at increased risk of disseminated candidiasis, nor are patients with congenital T-cell deficiencies (124, 138, 141).

Other Invasive Candidiasis Syndromes

Vaginal Candidiasis

Vulvovaginal candidiasis (VVC) is a common cause of presentation to a health care provider. As many as 75% of women have at least one episode of VVC during their lives (136), and up to 8% of those women have recurrent VVC (i.e., ≥4 episodes per year) (35). The overall economic impact of VVC on the U.S. health care system is substantial and has been estimated to be ≥$1 billion per year (34). Hence, VVC represents a potentially important market for vaccination.

Recurrent VVC is a potential specific disease target for vaccination, since the impact of an effective vaccine would be substantially greater in patients who develop recurrence after effective antifungal therapy than in patients who are cured with no relapse by antifungal therapy. A variety of host factors have been linked to increased risk of recurrent infection (136). Unfortunately, many of these risk factors are also immunomodulatory, such as HIV infection and corticosteroid therapy, and have the potential to blunt the efficacy of a vaccine. Genetic predispositions to recurrent VVC have been described, although the strength of association may be insufficient to use as a basis for clinical trial development (39). Diabetes mellitus is also a risk factor for VVC, but only if the diabetes is poorly controlled (136).

Treatment with antibiotics is the most promising risk factor to identify a high attack rate of VVC as a basis for enrollment into clinical trials of an active vaccine (32, 136, 145, 160). In one recent case control study, 25% of women treated for at least 3 days with an oral antibiotic developed symptomatic VVC by 4 to 6 weeks later (164). Other studies have also reported VVC attack rates of 25 to 33% in patients being treated with antibiotics (10, 116). These studies identify an immunocompetent patient population with a high attack rate of VVC which could be used to study the efficacy of a relevant vaccine in clinical trials, and would be a reasonable target population for vaccination postapproval. However, it must also be emphasized that the majority of patients who develop VVC have not been treated with antibiotics prior to the infection (136). Hence, a study focusing on antibiotic-associated VVC would encompass only a minority of the potential market need for a vaccine.

Pregnancy is also known to increase the frequency of symptomatic VVC (86, 111, 136). However, pregnant women would likely be excluded from vaccine clinical trials unless extremely detailed and convincing preclinical toxicity studies were available to provide reassurance of safety.

Of note, in contrast to disseminated candidiasis, which has witnessed a dramatic shift towards infections caused by non-*Candida albicans* species of *Candida*, VVC continues to be predominantly caused by *C. albicans* (136, 156). Hence, vaccination against VVC may not need to target non-*C. albicans* species as effectively as would an effective vaccine against disseminated infection.

A primary barrier to development of a vaccine targeting VVC is the confusion over the nature of protective adaptive immunity to VVC, as summarized by Fidel (32). Data from an unusual clinical study in which healthy volunteers were infected with *C. albicans* to cause VVC indicated that the nature of the innate response to the infection was the primary driver of symptomatic disease (33). If the innate immune cells were accompanied by significant inflammation, symptomatic disease was present, whereas the absence of inflammation correlated with a lack of symptomatic disease. Nevertheless, adaptive responses can regulate downstream innate immune responses, and in a murine model active vaccination resulted in diminished fungal burden in nonimmunocompromised mice (142). Hence, modulation of adaptive immune function by vaccination has the potential to be protective against VVC.

Oropharyngeal Candidiasis

In contrast to disseminated and vaginal candidiasis, oropharyngeal candidiasis (OPC) usually occurs in patients with substantial T-cell dysfunction, such as those with AIDS (130), and in patients receiving corticosteroid therapy (27). In one cross-sectional analysis, 15% of patients with HIV infection had OPC (129), and it is estimated that up to 90% of patients infected with HIV who do not receive effective antiretroviral therapy develop OPC (27). However, such infections may also be found in patients receiving local irradiation for head and neck cancers, where the predisposition is likely due to local anatomical defects caused by radiation rather than T-cell deficiency (122). Similarly, *Candida* is a common cause of denture stomatitis, again likely due to local trauma to the oral mucosa (27). OPC may also occur in patients being treated with inhaled steroids for asthma or other reactive airway diseases (14, 24). Finally, OPC may occur in patients with diabetes mellitus (27).

Development of a vaccine for OPC in patients with HIV/AIDS is complicated by the host immunosuppression and also by the fact that effective immune reconstitution therapy is now available for most patients. However, a vaccine for OPC may be feasible to develop for patients with predictable increases in acute risk of infection, such as patients in whom initiation of corticosteroid therapy or radiation therapy to the head and neck is anticipated to begin. Indeed, a murine model of OPC in which immunosuppression is achieved with corticosteroids demonstrated that vaccination could result in a dramatic decrease in fungal burden in the oropharynx despite the immunosuppression (142). Finally, vaccination to prevent denture stomatitis may be feasible.

Potential Barriers to Vaccine Efficacy for Invasive Candidiasis

Immunosuppression in At-Risk Patients

As mentioned above, disseminated candidiasis and OPC are opportunistic infections, which do not typically affect patients lacking specific risk factors. In contrast, vaginal candidiasis affects women with no known specific risk factors, although subtle alterations in host immunity may be present in patients with recurrent VVC. The presence of immunodeficiency in a minority of patients at risk for disseminated candidiasis and in the majority of patients who develop

OPC may be a barrier to development and deployment of a vaccine against invasive candidal syndromes.

It must be emphasized that immunocompromised patients (e.g., neutropenic patients and those receiving steroids) are the minority of patients at risk for disseminated candidiasis. The majority of patients at risk for disseminated candidiasis are those undergoing emergent or elective gastrointestinal or cardiac surgery (e.g., appendectomy, cholecystectomy, cardiac bypass, or valve repair), those with central venous catheters, those receiving broad-spectrum antibiotics, or in general those patients receiving their care in intensive care units, including both civilian and military trauma patients receiving abdominal, chest, or burn wounds. These populations of nonneutropenic, susceptible patients are enormous, collectively numbering in the millions per year in the United States and all technically advanced countries.

Furthermore, the diminished immune responsiveness to *Candida* in immunocompromised patients actually underscores the need to vaccinate such patients, rather than providing a rationale for not vaccinating them. While it is clear that the response to vaccines of patients with altered cell-mediated immunity is blunted, a significant fraction of immunocompromised patients respond to a variety of vaccines. For example, administration of the 23-valent unconjugated Pneumovax and a 5-valent conjugated vaccine against *Streptococcus pneumoniae* resulted in, respectively, 31 and 60% response rates (defined as fourfold rises in antibody titers) in HIV-infected children (65). Kroon et al. reported a >60% response to Pneumovax in HIV-infected adults (70). Similarly, in several studies administration of the conjugated *Haemophilus influenzae* type b (Hib) vaccine resulted in 40% response rates in HIV-infected adults and children (17, 25, 127). Even more impressive was the finding that administration of a booster dose of the Hib vaccine increased the response rate to 57%. Using a different definition of vaccine response (>1-μg/ml absolute antibody titer, thought to be protective), Dockrell et al. reported that administration of Hib to adults infected with HIV resulted in an impressive 90% response rate (26). Similar results were found in a cohort of HIV-infected children in the United States (121). Viral vaccines are also immunogenic in patients infected with HIV. Levin et al. found that 60% of HIV-infected children seroconverted after administration of the varicella vaccine, and 83% had positive lymphocyte proliferation assays (77). All 10 HIV-positive patients inadvertently administered the smallpox vaccine were found to have normal "take" reactions (149). Finally, both hepatitis A and B vaccines mediate significant immune responses in HIV-infected patients (63, 150).

In most studies, the magnitude of the vaccine responses was blunted in HIV-infected patients compared to healthy controls. Nevertheless, the vaccines did result in significant and detectable responses in HIV-infected patients. Indeed, clinical trials have demonstrated that these vaccines not only are immunogenic but also reduce the burden of *H. influenzae* and *S. pneumoniae* invasive diseases by up to 70% in HIV-infected patients (44, 67, 84).

It must also be noted that despite reduced vaccine efficacy in immunocompromised patients, if the target disease is more frequent in compromised hosts, it is possible for vaccines to prevent a greater burden of disease in compromised hosts than in competent hosts. For example, Madhi et al. recently published their experience administering the 9-valent conjugated pneumococcal vaccine to children in Africa (42, 83). They found that the vaccine resulted in a 20% relative reduction in clinical pneumonia in HIV-negative children, compared to a 15% relative reduction in HIV-infected children. However, due to the increased frequency of pneumonia in children infected with HIV, on a population basis the vaccine was 10-fold more effective at preventing pneumonia in HIV-infected children than in HIV-negative children (vaccine attributable reduction = 2,302 versus 267 cases per 100,000 child-years for HIV-infected versus uninfected children). Therefore, the concept that immunocompromised patients can be protected from invasive infection by immunization has been well validated and extensively documented in both immunogenicity and clinical outcomes studies.

Also in support of the current application are studies demonstrating significant vaccine responses in patients with neutropenia from leukemia or cancer chemotherapy. For example, Sinsalo et al. demonstrated a 43% response rate to the Hib vaccine in elderly patients with leukemia (134). Feldman et al. reported that 50% of children with acute leukemia responded to the Hib vaccine and that the response rate was 75% if the vaccine was administered during the first year of chemotherapy (31). Of note, this vaccine responsiveness was demonstrated despite the neutropenia resulting from the children's leukemia or chemotherapy. A similar percentage (>70%) of patients with solid cancers or lymphomas undergoing chemotherapy responded to both the influenza vaccine and Pneumovax (103). Leung et al. administered live-attenuated varicella vaccine to children with leukemia or solid tumors and found a marked increase in T-cell proliferation to the virus and a 94% seroconversion rate after two doses (76). Again, this response rate was seen despite the presence of neutropenia in these patients from their leukemia and/or chemotherapy. Similarly, a hepatitis B vaccine resulted in a seroconversion rate of 90% of children with acute lymphoblastic leukemia and significantly reduced the incidence of hepatitis B virus infection in this cohort during long-term follow-up (137). Because of these and other data, it is specifically recommended to administer vaccines to patients with cancer despite their immunocompromised condition (5). Therefore, the suppression of cell-mediated immunity in populations at risk for *Candida* infections is actually a specific reason to vaccinate those patients.

Human Preimmunity to *Candida*

Because *Candida* colonizes a significant percentage of human beings, some degree of preexisting immunity to the organism is expected in most individuals. Approximately 50 to 70% of healthy controls react to *Candida* antigens when they are administered intradermally (3, 7, 16, 20, 97, 105). Although *Candida* colonization clearly leads to skin test reactivity in humans, the hypothesis that the resultant immunity to the organism is protective is not supported by the available literature. If this hypothesis were correct, colonized patients would be relatively protected from developing disseminated candidiasis. In fact, the exact opposite is true; it is widely recognized that colonization with *Candida* spp. is a major independent risk factor for the development of disseminated candidiasis (109, 113). Hence, while it is recognized that patients colonized with *Candida* often have delayed-type hypersensitivity (DTH) to the organism (i.e., are skin test positive), this immunity is not sufficient to protect patients from developing disseminated disease.

Individuals with a degree of preexisting immunity to the fungus are likely to derive benefit from vaccination. The general response to repeated exposure to an antigen is increased immune responsiveness over time (112). This

observation forms the very basis for the modern practice of vaccination and the practice of booster doses for vaccines. Therefore, the kinetics and magnitude of immunity to *Candida* are likely to be favorably altered by vaccination of preimmune individuals. Specific evidence in support of the ability of vaccination to enormously decrease disease in populations already colonized with and preimmune to the targeted pathogen derives from the experience with *Haemophilus influenzae*. The conjugated Hib vaccine has decreased invasive disease due to *H. influenzae* by more than 95% in the United States (114). This vaccine efficacy has occurred despite the fact that, akin to the case with *Candida*, both children and adults had significant oropharyngeal colonization rates (up to 60% in some studies) of *H. influenzae* in the prevaccine era (6, 157, 166). These colonized patients were known to have detectable levels of antibody against *H. influenzae* (8, 75). Nevertheless, vaccination successfully increased titers of antibody to the organism, increased the rate of eradication of colonization, and decreased subsequent invasive disease (30, 85).

The precise same situation is true of *S. pneumoniae*. Up to half of unvaccinated children are colonized with *S. pneumoniae* (11, 12, 23, 40, 81), many of whom also have detectable type-specific immunoglobulin G (IgG) responses (41, 78). Unvaccinated adults are also colonized, albeit at somewhat lower rates (72, 99). This colonization results in production of type-specific antibodies which offer incomplete protection against subsequent infection (40, 98–100, 120). Vaccination results in an increase in serotype reactivity to nearly 80% (99), a decrease in colonization in both children (146) and adults (126), and a marked decrease in the incidence of invasive pneumococcal disease (9, 13, 38, 68, 131, 132, 135). Therefore, based on the fundamental practice of vaccine boosting and the parallel experience with both *H. influenzae* and *S. pneumoniae*, vaccination is likely to be effective even in patients who are preimmune to *Candida*.

PASSIVE IMMUNIZATION STRATEGIES AGAINST INVASIVE CANDIDIASIS

Primary Use of Passive Immunization against Invasive Candidiasis

In contrast to active immunization, for which the primary target population consists of acutely at-risk patients who are not yet infected, passive immunization for invasive candidal infections is more likely to be developed for patients with invasive candidiasis. Passive immunotherapy is typically far more expensive than administration of an active vaccine. Hence, passive immunotherapy is unlikely to be cost-effective in a prophylactic setting, where it would have to be administered to many patients to prevent one case of infection. Furthermore, the substantial efficacy of antifungal therapy for both OPC and vaginal candidiasis, and the relatively low morbidity rate and extremely low mortality rate resulting from these infections, likely precludes deployment of adjunctive passive immunotherapy for these infections. Hence, the primary potential of passive immunization is as adjunctive immunotherapy, in addition to antifungal agents, for patients with disseminated candidiasis.

Three predominant targets have been the focus of passive immunization strategies against disseminated candidiasis: (i) antimannan antibiodies, (ii) antiglucan antibodies, and (iii) anti-heat shock protein antibody. Each of these strategies is reviewed.

Antimannan Passive Immunization

Mannan is the predominant constituent of the external facing cell wall of *Candida* spp. (101). Mannan exists in the cell wall in the form of mannoproteins or complex mannan polysaccharide networks. Given its ubiquitous presence on the candidal cell surface, efforts to develop passive immunotherapy against disseminated candidiasis logically focused on antimannan antibodies. James Cutler and colleagues have been developing antimannan antibodies as immunotherapy for disseminated candidiasis. Over 15 years ago Han and Cutler described the use of passive immunization with antibodies raised against liposomally encapsulated candidal mannan to protect recipient mice from disseminated candidiasis (46). From the immunization, an IgM monoclonal antibody, B6.1, was found to be specific for mannan and to protect both normal and SCID mice against disseminated candidiasis. The B6.1 antibody was found to recognize a β-1,2 mannotriose epitope spread diffusely across the candidal cell surface, to react only with blastospores and not hyphae (47), and to serve as an effective opsonin, enhancing neutrophil ingestion and killing of *C. albicans* blastospores (15). Of note, passive immunization with the B6.1 monoclonal antibody was also found to reduce by 60 to 80% the vaginal fungal burden in a nonimmunocompromised model of murine candidal vaginitis (48, 50). A second monoclonal antibody raised against the same epitope but with an IgG3 Fc isotype was also found to be protective against both disseminated and vaginal candidiasis (49). Complement activation appeared to be required for the efficacy of the passive immunization (21).

The results of these passive immunization experiments in mice are encouraging. Whether such antibodies will be capable of being developed in a humanized form for clinical use is not yet clear.

Antiglucan Passive Immunization

In contrast to mannan, which predominantly exists on the outer surface of the candidal cell wall, β glucan is a predominant constituent of the internal aspect of the cell wall (101). Nevertheless, antiglucan antibodies are capable of binding to the candidal cell surface, even in the absence of heat or other agents which increase β glucan exposure on the cell surface. The lead strategy for developing antiglucan passive immunotherapy has been based on a protein conjugate vaccine consisting of algal glucan linked to diphtheria toxoid as a carrier protein. Passive immunization with serum induced by this vaccine resulted in significant protection against both disseminated and vaginal candidiasis in a murine model (151). Remarkably, the antibodies were shown to directly inhibit the growth of *C. albicans* in vitro, and to also inhibit the growth of *Aspergillus fumigatus* in vitro, likely due to the presence of glucan in the *A. fumigatus* cell wall. In a follow-up study, the antibodies were also shown to inhibit the growth of *C. neoformans* in vitro (118), indicating the potential breadth of efficacy of an antiglucan antibody passive immunization strategy.

In a follow-up study focusing on the mechanism of the antiglucan antibodies, an IgM and IgG2b monoclonal antibody were shown to bind to *C. albicans* β glucan. The specific epitope targeted by both antibodies was β-1,3–β-1,6 glucan in the fungal cell wall. Only the IgG2b antibody was protective during both disseminated and vaginal candidiasis. The IgG2b antibody bound to β-1,3 glucan, in contrast to the IgM antibody, which bound to β-1,6- and β-1,4-linked glucan, as well as β-1,3-linked glucan. In contrast to antimannan antibodies, the protective antiglucan antibody

did not enhance opsonophagocytic killing of C. *albicans* but did directly inhibit hyphal growth and adherence to human epithelial cells. Hence, like antimannan antibodies, antiglucan antibodies are a promising strategy to develop as adjunctive therapy for disseminated candidiasis. While antibody-based therapy for vaginal candidiasis is likely not practical, the fact that the same vaccine and antibodies (both antimannan and antiglucan) were able to induce protection against both disseminated and vaginal candidiasis suggests that active vaccine strategies may be able to protect against both infections as well.

Anti-Heat Shock Protein Passive Immunization

After many years of development, an anti-heat shock protein monoclonal antibody has entered clinical testing. The original observations by Matthews et al. regarding the potential of an anti-heat shock protein-based immunotherapy strategy were published in 1987 and in 1991 (90, 91). The investigators reported that patients recovering from disseminated candidiasis developed selective antibodies targeting heat shock protein 90 (HSP90) from C. *albicans*. Furthermore, transfer of serum from two of the convalescing patients improved the survival of mice subsequently infected with disseminated candidiasis. Finally, a murine monoclonal antibody targeting the LKVIRK epitope from candidal HSP90 was protective during murine disseminated candidiasis.

These results formed the basis for a subsequent clinical development program focusing on a humanized anti-HSP90 monoclonal antibody. The humanized antibody was shown to be protective against disseminated candidiasis in murine studies (89). The humanized antibody, efungumab (Mycograb), was shown to directly result in growth inhibition and killing of C*andida* spp. in vitro, including C. *albicans*, C. *glabrata*, C. *krusei*, C. *tropicalis*, and C. *parapsilosis*. Furthermore, Mycograb was synergistic with amphotericin B deoxycholate (AmB) in vitro. Mycograb also reduced tissue fungal burden when administered to mice subsequently infected with disseminated candidiasis, and it was synergistic in vivo with AmB, enhancing reduction of tissue fungal burden (92). In a follow-up study, Mycograb was found to synergistically improve the fungicidal effects of the echinocandin caspofungin against C*andida* spp. (53).

Subsequently, a multicenter, randomized, placebo-controlled comparative study of liposomal amphotericin (LAmB) alone versus LAmB plus efungumab was conducted in 139 patients with invasive candidal syndromes (107). Of these patients, 53 in the placebo arm and 44 in the treated arm had candidemia or a culture from a normally sterile site. Of note, there were more patients with neutropenia or AIDS in the placebo arm ($n = 6$) than in the efungumab arm ($n = 1$; $P = 0.07$ by Fisher's exact test). Otherwise the patients were well matched. Approximately 65% of the infections were caused by C. *albicans*, with the remainder divided among C. *glabrata* (7%), C. *tropicalis* (5%), C. *parapsilosis* (5%), and a mixture of others. The primary efficacy analysis was conducted at day 10 after initiation of therapy and found a complete overall response rate of 84% versus 48% in the efungumab versus placebo arm ($P < 0.01$). Furthermore, the attributable mortality rate at 33 days was 18% in the placebo arm versus 4% in the efungumab arm ($P = 0.025$). Efungumab also shortened the time to sterilization of cultures. However, 50% of patients in the placebo group never sterilized their cultures, which is highly discordant from the results of prior clinical trials of antifungal therapy for disseminated candidiasis. Finally, the antibody was well tolerated in general, with an adverse-effect profile similar to that in the control arm.

In general, the study results were strongly supportive of the concept of adjunctive passive immunotherapy in patients being treated for disseminated candidiasis. However, there were concerns regarding the study design and manufacture of the product, and neither the U.S. Food and Drug Administration (FDA) nor the European Medicines Agency approved the molecule for use in humans based on the clinical trial. NeuTec Pharmaceuticals, which originally owned Mycograb, has since been purchased by Novartis, which is actively working to develop appropriate manufacturing techniques to support a follow-up phase III clinical trial. In the meantime, Mycograb has demonstrated the promise and feasibility of a monoclonal antibody-based adjunctive immunotherapy strategy for invasive fungal infections.

ACTIVE IMMUNIZATION STRATEGIES AGAINST INVASIVE CANDIDIASIS

β Glucan-Conjugated and Other Vaccines

The aforementioned passive immunization strategy focusing on β glucan has been based upon raising protective antibodies by actively vaccinating with β glucan conjugated to a carrier protein. Initially, the β-1,3 glucan laminarin from the brown alga *Laminaria digitata* was conjugated to diphtheria toxoid and used to immunize mice with Freund's adjuvant (151). Vaccinated mice had improved survival compared to control mice after intravenous (i.v.) challenge (75% versus 15% survival for vaccinated versus control mice at 35 days of follow-up). Vaginal administration of the vaccine with cholera toxin somewhat increased the rate of clearance of C. *albicans* in a rat model of candidal vaginitis. As mentioned previously, the mechanism of protection appeared to be induction of antibodies which directly inhibited fungal growth.

Of note, antibodies induced by the laminarin-diphtheria toxin conjugate vaccine were found to bind to the surface of *Aspergillus fumigatus* and inhibit its growth. Subsequently, the efficacy of active vaccination was tested in a murine model of i.v. infection with *A. fumigatus*. Remarkably, the vaccine was found to improve survival in such mice. Nevertheless, in order for the laminarin-diphtheria toxin conjugate to be developed into a clinically testable vaccine, active vaccination with it will have to achieve significant efficacy with use of an alternative adjuvant, as Freund's adjuvant is too toxic for use in humans.

Other anticandidal vaccines that have been assessed in published preclinical studies have focused on immunizing with candidal heat shock proteins (17) or protein extracts of the candidal cell wall (18). However, critical developmental milestones with such vaccines, such as achievement of efficacy against an infectious challenge with an adjuvant that can be used in humans, have not yet been published. Nevertheless, studies continue to build upon the fundamental knowledge of protective immunity against *Candida*, which should ultimately lead to development of a clinically useful vaccine.

Als Vaccines

The candidal vaccine under development at Harbor-UCLA Medical Center appears to be furthest along the developmental pathway. The C. *albicans ALS* family is comprised of at least eight genes (55, 56). In determining the potential

for an Als family member to serve as a useful active vaccine candidate, heterologous expression was used to investigate the ability of Als proteins to mediate adherence to various substrates to which *C. albicans* is known to bind (29, 64, 66, 93). *Saccharomyces cerevisiae* strain S150-2B transformed with *ALS1*, *-3*, *-5*, *-6*, *-7*, or *-9* open reading frames under the control of the constitutive *ADH1* promoter mediated adherence to strikingly different substrates (133). While Als1p and Als3p were promiscuous in their substrate affinity, other Als family members had more restrictive adhesive functions. Because Als1p and Als3p appear to be the most promiscuous of the Als family members, mediating high-level adherence to the broadest array of substrates, initial efforts at vaccine development focused on them.

For an effective vaccine, the target protein should be conserved among *C. albicans* strains. Therefore, we cloned and sequenced the N-terminal regions of *ALS1* genes in a diverse group of clinical isolates from bloodstream (5 strains), urine (5 strains), and oropharyngeal (10 strains) infections. Sequence analysis of these N termini revealed that the N-terminal amino acid sequences of all isolates tested are >99.9% identical. Additionally, it has been reported that the *ALS* gene family is present in many *Candida* species, including *C. dubliniensis* and *C. tropicalis* (56). An adhesin analogous to Als family members has been described for *C. glabrata* (19, 37). This conservation of Als sequences in *C. albicans* isolates and among different species underscores the feasibility of using this family of proteins in generating an effective vaccine against infections caused by multiple strains and multiple species of *Candida*.

Based on the above results, we explored the effect of immunization of mice with rAls1p-N on the outcome of hematogenously disseminated candidiasis. rAls1p-N (from amino acid 17 to 432) produced in *S. cerevisiae* was purified by His tag binding followed by gel filtration and Ni-nitrilotriacetic acid matrix affinity purification. In initial experiments on immunogenicity and protection, female BALB/c mice were vaccinated by intraperitoneal injection of rAls1p-N mixed with complete Freund's adjuvant (CFA) at day 0 and boosted with another dose of the antigen with incomplete Freund's adjuvant (IFA) at day 21. To explore the strength of the humoral immune response following active immunization with rAls1p-N, mice were bled 2 weeks after boosting and anti-Als1p titers were measured by enzyme-linked immunosorbent assay plates coated with the N terminus of Als1p. Subsequently, these mice were infected with *C. albicans* and the survival of mice was compared with the antibody titer (58). When the serum antibody titers of individual mice were plotted against the survival time of each mouse, no correlation between antibody titer and survival was found (58). Indeed, mice with antibody titers in excess of 1:100,000 had survival durations no different from those of mice with titers at the lower limit of detection (~1:100).

As humoral immunity did not correlate with rAls1p-N-induced protection, the cell-mediated immune response induced by protective and nonprotective doses of rAls1p-N was evaluated. Mice were immunized as described above. Two weeks after the boost, splenocytes were harvested and cultured in the presence of heat-killed, pregerminated *C. albicans*. Following 48 h of culture, splenocytes were harvested for intracellular cytokine detection by flow cytometry. Only lymphocytes from mice immunized with the protective dose of antigen developed a significantly increased frequency of Th1 cells compared to that in mice given adjuvant alone ($P = 0.03$) (58). To confirm that type 1 immunity was stimulated by r-Als1p-N in vivo, DTH was tested by footpad swelling. Only mice vaccinated with the protective dose of rAls1p-N developed a significantly increased DTH reaction compared to that in controls ($P < 0.05$ for all comparisons versus the 20-μg dose) (58).

Finally, to define the role of antibody and T cells in vaccine-mediated protection, B-cell-deficient, T-cell-deficient nude, or congenic BALB/c wild-type control mice were immunized as described above and infected with a lethal inoculum of *C. albicans*. B-cell-deficient mice trended to being more resistant, whereas T-cell-deficient mice were more susceptible to infection than wild-type control mice given adjuvant alone (58). The rAls1p-N vaccine maintained its efficacy in B-cell-deficient mice but was ineffective in T-cell-deficient mice (58). Thus, protection of rAls1p-N against *C. albicans* required T cells. In contrast, B-cell function was dispensable.

To determine the influence of route of administration, we and others tested the efficacy of rAls1p-N administered subcutaneously (s.c.). Female BALB/c mice were vaccinated s.c. with CFA on day 0, boosted in IFA on day 21, and infected via the tail vein 14 days later with the highly virulent clinical isolate *C. albicans* SC5314. Immunizing s.c. with rAls1p-N resulted in 50 to 60% long-term survival of mice infected with otherwise 100% fatal inocula of *C. albicans* (143). We have determined that the cause of death in this murine model of disseminated candidiasis is progressive septic shock (139). Furthermore, the inocula used in our vaccine challenge studies were 25- to 50-fold above the 50% lethal dose of *C. albicans* in the murine model. Hence, the challenge was very rigorous, and vaccination resulted in similar survival in the murine model of candidal sepsis as does antifungal therapy in clinical cases of candidal sepsis (73, 106).

Given the known sequence conservation of the Als1 gene across clinical isolates, we hypothesized that the rAls1p-N vaccine would be effective against multiple strains of *C. albicans*. Indeed, when BALB/c mice vaccinated with rAls1p-N plus CFA or CFA alone were infected via the tail vein with other strains of *C. albicans*, vaccine efficacy was retained (57). Finally, the efficacy of rAls1p-N was tested in BALB/c mice infected via the tail vein with *C. glabrata*, *C. krusei*, *C. parapsilosis*, or *C. tropicalis*. Because these species do not cause lethal infection in mice, tissue fungal burden was measured on day 5 postinfection to assess vaccine efficacy. The rAls1p-N vaccine reduced kidney fungal burden against all species tested (57).

To determine the potential for an Als3-based vaccine, the recombinant N terminus of Als3p (rAls3p-N) was expressed in *S. cerevisiae* and purified by Ni-agarose affinity purification, in the same manner as described for rAls1p-N. In our initial experiments, BALB/c mice were given CFA alone, CFA plus rAls1p-N (20 μg), or CFA plus rAls3p-N (20 μg), with booster doses 3 weeks later. Mice vaccinated with either rAls1p-N or rAls3p-N developed titers of anti-rAls1p-N antibodies significantly greater than those of mice receiving CFA alone (142). Of note, mice vaccinated with rAls3p-N generated anti-rAls1p-N antibodies at titers equivalent to those of mice vaccinated with rAls1p-N. In contrast, mice vaccinated with rAls1p-N generated much less impressive titers against rAls3p-N than did mice vaccinated with rAls3p-N. Thus, rAls3p-N induces a more broadly cross-reactive antibody response than rAls1p-N. Next, BALB/c mice were vaccinated with CFA, CFA plus rAls1p-N, or CFA plus rAls3p-N and infected via the tail vein with *C. albicans*. Both vaccines resulted in significant

improvement in survival compared to that of mice receiving adjuvant alone (142). Also, the efficacies of the two vaccines were similar.

Because Als3p mediates superior adhesion to epithelial cells compared to Als1p, we and others hypothesized that rAls3p-N might have unique efficacy in mucosal infection. We therefore compared rAls1p-N versus rAls3p-N in our steroid-treated, oropharyngeal model of infection and in a model of candidal vaginitis. In a nonimmunocompromised model of candidal vaginitis, the rAls3p-N vaccine mediated a median 0.7-log CFU/g decrease in vaginal fungal burden compared to CFA (142). In contrast, rAls1p-N mediated no benefit at all in the vaginitis model, and rAls3p-N was significantly more effective than rAls1p-N.

In cortisone-treated mice with OPC, the rAls1p-N vaccine mediated a trend towards reduced tongue fungal burden, while the rAls3p-N vaccine significantly reduced tongue fungal burden (142). By histopathology, both rAls1p-N and rAls3p-N mediated marked reductions in the severity of tissue invasion and inflammatory response compared to adjuvant alone (142).

Because Freund's adjuvant is too toxic for use in humans, we sought to determine if another adjuvant would be effective in combination with rAls3p-N. We therefore combined rAls3p-N (300 μg) with 0.1% Alhydrogel [aluminum hydroxide gel, $Al(OH)_3$] in phosphate-buffered saline (PBS) and compared its efficacy to that of $Al(OH)_3$ alone. In the initial experiment, we also compared rAls3p-N efficacy with that of a distantly related Als family member, rAls5p-N produced in the same *S. cerevisiae* expression vector and purified by the same methods. We challenged mice with a rapidly lethal inoculum and found that the rAls3p-N vaccine was effective but rAls5p-N was not (140).

To determine the impact of boosting on efficacy in survival studies, BALB/c mice were vaccinated with one or two doses of $Al(OH)_3$ alone versus $Al(OH)_3$ plus rAls3p-N. Mice were then infected via the tail vein with *C. albicans* SC5314 or 15563. Protection against the 15563 isolate was similar regardless of whether one or two doses of vaccine were administered, and protection approached 100%. Against the SC5314 isolate, mice receiving two doses of vaccination had a trend to improved survival compared to mice receiving one dose of vaccine (140). Collectively, these data indicate that significant protection is afforded after only one dose of vaccine.

The immunogenicity of rAls3p-N was tested by vaccinating mice with PBS-$Al(OH)_3$ (P-A), PBS-rAls3p-N-$Al(OH)_3$ (P-3), saline-$Al(OH)_3$ (S-A), or saline-rAls3p-N-$Al(OH)_3$ (S-3). Three weeks after vaccination, splenocytes were harvested and stimulated with rAls3p-N or tetanus toxoid (negative control) ex vivo. Surprisingly, rAls3p-N stimulation of splenocytes from P-3-vaccinated mice generated significantly higher frequencies of Th1 and Th2 cells than all other groups (79). Similarly, supernatant from rAls3p-N-stimulated splenocytes from P-3-vaccinated mice contained significantly elevated levels of tumor necrosis factor (TNF), gamma interferon (IFN-γ), IL-4, and IL-5 compared to supernatant from P-A splenocytes (79). Supernatant from S-3-vaccinated splenocytes contained significantly elevated levels only of IL-4 and IL-5 compared to supernatant from S-A splenocytes. Supernatant from P-3 splenocytes contained significantly more TNF and IFN-γ, and a trend to more IL-4, than supernatant from S-3 splenocytes. P-3 splenocyte supernatant also had the highest IFN-γ/IL-4 ratio of all groups. These results confirmed that use of $Al(OH)_3$ as adjuvant enabled priming of proinflammatory, Th1 lymphocytes, which are hypothesized to be critical to vaccine-mediated protection.

To determine the importance of type 1 versus type 2 cytokines, vaccine efficacy was compared in mice congenitally deficient in IL-4 or IFN-γ versus wild-type controls. Mice deficient in IFN-γ were significantly more susceptible to candidemia than wild-type mice (140); mice deficient in IL-4 were susceptible similarly to wild-type mice (140). Vaccination with rAls3p-N was as effective in IL-4-deficient mice as in wild-type controls. Vaccination marginally improved survival of mice with IFN-γ deficiency, but vaccinated IFN-γ-deficient mice had significantly worse survival than wild-type control mice. These data demonstrate that IFN-γ but not IL-4 is required for host defense against disseminated candidiasis and that IFN-γ but not IL-4 is required for vaccine-induced protection.

To confirm the role of T cells in rAls3p-N vaccine-induced protection against disseminated candidiasis, BALB/c mice were vaccinated with rAls3p-N plus $Al(OH)_3$ or with $Al(OH)_3$ alone. Two weeks following the boost, splenocytes were harvested as previously described (144). $CD3^+$, $CD4^+$, or $CD8^+$ T lymphocytes or $B220^+$ B lymphocytes were purified (>95%) by using BD IMag particles, per the manufacturer's instructions. In addition, serum was harvested from immune or control mice by terminal bleed. A total of 10^7 $CD3^+$ T or $B220^+$ B lymphocytes, or 5×10^6 $CD4^+$ or $CD8^+$ T lymphocytes, were adoptively transferred, via i.v. injection, into congenic BALB/c mice that had not been vaccinated. The next day, 250 μl of immune or control serum was administered to other BALB/c mice. Recipient mice were infected via the tail vein with *C. albicans* SC5314 (2.5×10^5) 3 h after serum administration and 24 h after administration of immune or control lymphocytes. $CD3^+$, $CD4^+$, or $CD8^+$ T cells from vaccinated but not control donors transferred protection to naïve recipient mice (140). Survival of mice receiving $CD4^+$ splenocytes from vaccinated mice was greater than survival of $CD8^+$ splenocyte recipients (140). In contrast, $B220^+$ cells did not transfer protection, nor did immune serum.

Having demonstrated that $CD4^+$ T cells transferred protection, and that IFN-γ was required for protection, our group next sought to determine if the IFN-γ production from lymphocytes or other cellular effectors was required. We adoptively transferred $CD4^+$ lymphocytes from vaccinated or control wild-type mice into IFN-γ-deficient recipient mice, or from IFN-γ-deficient donor mice into wild-type recipient mice. We found that transfer of $CD4^+$ cells from wild-type mice into IFN-γ-deficient mice transferred protection but that transfer of $CD4^+$ cells from IFN-γ-deficient donor mice into wild-type recipient mice did not transfer protection (80). These data are consistent with a requirement for protection of upstream IFN-γ production by $CD4^+$ cells responding to the vaccine.

To determine if phagocyte function is required for rAls3p-N vaccine efficacy, we obtained $gp91^{phox-/-}$ mice, which are used as a model for chronic granulomatous disease. Initially our group performed pilot studies to determine how susceptible to disseminated candidiasis these $gp91^{phox-/-}$ mice are. Of note, we found that the 100% lethal dose of *C. albicans* SC5314 was 250-fold lower in $gp91^{phox-/-}$ mice than in wild-type controls (10^3 versus 2.5×10^5) (80). These data confirm that production of oxygen free radicals is of critical importance to murine host defense against disseminated candidiasis.

Subsequently, $gp91^{phox-/-}$ and wild-type mice were vaccinated with rAls3p-N (300 μg) plus $Al(OH)_3$ or $Al(OH)_3$

alone. $CD4^+$ T cells from some of the vaccinated wild-type mice were adoptively transferred into $gp91^{phox-/-}$ recipient mice, and vice versa. The vaccine provided no significant protection against infection in $gp91^{phox-/-}$ mice. Furthermore, $CD4^+$ splenic lymphocytes from vaccinated $gp91^{phox-/-}$ mice transferred protection to wild-type recipient mice. In contrast, $CD4^+$ splenic lymphocytes from vaccinated, wild-type donor mice failed to transfer protection to $gp91^{phox-/-}$ recipient mice (80). These data demonstrate that abrogation of superoxide production blocks the ability of $CD4^+$ lymphocytes from vaccinated animals to transfer immune protection in recipient mice. Hence, these data confirm that rAls3p-N-responsive lymphocytes require functional phagocytes to provide protection against disseminated candidiasis.

The need for downstream functional phagocytes to mediate vaccine protection suggested that Th17 cells, which are known to act by recruiting phagocytes to the sites of infection (147, 165), might play a role in vaccine protection. To determine the requirement for IL-17 and Th17 cells in vaccine-mediated protection, our group vaccinated mice deficient in IL-17A, or their wild-type congenic control mice. IL-17A deficiency abrogated vaccine-mediated protection. In contrast to IFN-γ deficiency, IL-17A deficiency did not exacerbate infection in unvaccinated mice (survival of unvaccinated deficient versus wild-type mice) (80).

To determine if $CD4^+$ T cells were the primary source of IL-17A in mediating vaccine-induced protection, $CD4^+$ T cells from vaccinated or control mice were cross-adoptively transferred into recipient mice (IL-17A-deficient donor cells transferred to wild-type recipient mice and wild-type donor cells transferred to IL-17A-deficient recipient mice). We also repeated the survival study in wild-type and IL-17-A-deficient mice that did not undergo adoptive transfer to serve as positive and negative controls for the adoptive-transfer study. Mice were infected the day after adoptive transfer. Once again, the vaccine protected the positive control wild-type mice but not the negative control IL-17-A-deficient mice. Adoptive transfer of $CD4^+$ cells from vaccinated wild-type to IL-17A-deficient recipient mice transferred protection against infection (80). In contrast, transfer of $CD4^+$ T cells from vaccinated IL-17A-deficient donor mice to wild-type recipient mice failed to transfer protection, confirming that $CD4^+$ T-cell-derived IL-17A was necessary to mediate vaccine-induced protection.

To define the populations of cells induced by vaccination, spleens and lymph nodes were harvested from vaccinated and control mice 2 weeks following the boost. The cells were stimulated ex vivo for 5 days with rAls3p-N. Analysis of supernatants by cytometric bead array confirmed that cells from vaccinated mice produced significantly more IFN-γ and IL-17, as well as the neutrophil-acting CXC chemokines, KC and MIP-1α, than did cells from control mice. IL-10 levels were also significantly higher in supernatants from vaccinated than control mice. IL-4 levels were not detectable in any supernatant from control cells; IL-4 was detectable at low levels (<2 μg/ml) in supernatants from four of the eight mice in the vaccinated group. Transforming growth factor β and IL-6 levels were low and not significantly different in supernatants from vaccinated or control mice. Supernatants from stimulated immune cells markedly enhanced phagocytic killing of *C. albicans* ex vivo compared to supernatant from control cells. Finally, intracellular cytokine analysis of the stimulated cells demonstrated that vaccination resulted in increased frequencies of Th1 ($CD4^+$ IFN-γ^+), Th17 ($CD4^+$ IL-17^+), and Th1/17 ($CD4^+$ IFN-γ^+ IL-17^+) cells in draining lymph nodes and splenocytes (data not shown due to space limitations) compared to the frequencies in unvaccinated mice. Vaccination resulted in approximately a 10-fold reduction in kidney fungal burden and a marked increase in neutrophil influx relative to the infectious burden of organism in the tissues. Concordant with ex vivo cytokine measurements, levels of IFN-γ, IL-17, and the CXC chemokine KC were higher in the kidneys of vaccinated than of control mice.

Establishment of efficacy in combination with an aluminum-based adjuvant is a critical milestone, because aluminum derivatives have been used in FDA-approved vaccines for more than half a century. Hence, a dosing schedule, route of administration, and adjuvant have now been identified for rAls3p-N that can support an Investigational New Drug (IND) application to enable clinical testing to begin, after Good Manufacturing Practice (GMP)-compliant manufacturing is achieved.

Indeed, based on the magnitude of the efficacy data available, and the fact that efficacy is retained with aluminum hydroxide adjuvant, the vaccine is currently undergoing manufacturing development in support of a future IND for a phase I clinical trial. The costs of preparation for an IND are significant and include several million dollars just to develop GMP-compliant manufacturing, as well as additional costs for preclinical toxicity studies using GMP-compliant material, not including the costs of clinical protocol development. These costs represent a major barrier to development of vaccines for invasive fungal infections in general and are an even greater barrier for infections caused by organisms other than *Candida*, which have smaller perceived markets driving investment capital.

CONCLUSIONS

With the aging global and U.S. populations, and increasingly intensive medical treatments of critical illnesses, the incidence of invasive fungal infections will continue to rise over the coming decades. Mortality rates associated with these diseases remain high, despite the availability of new antifungal agents. Hence, prevention of these infections by vaccination is highly desirable. Due to enhanced understanding of host defense and pathogenetic mechanisms that underlie invasive fungal infections, we are now in a position to begin developing such vaccines. The concept of niche vaccination of acutely at-risk patients or patients in restricted geographical areas is a new idea in vaccinology. The lack of complete understanding of the market potential for such vaccines has created significant impediments to the availability of the requisite capital to develop these vaccines. Continued education about the economic importance of vaccines for invasive fungal infections, combined with continued development of well-defined antigens and effective adjuvants with a track record of safety, should enable such vaccines to enter clinical testing in the coming decade.

We own equity in NovaDigm Therapeutics, Inc., which is developing candidal vaccine technologies.

REFERENCES

1. **Alonso-Valle, H., O. Acha, J. D. Garcia-Palomo, C. Farinas-Alvarez, C. Fernandez-Mazarrasa, and M. C. Farinas.** 2003. Candidemia in a tertiary care hospital: epidemiology and factors influencing mortality. *Eur. J. Clin. Microbiol. Infect. Dis.* **22:**254–257.

2. **Anaissie, E. J., J. H. Rex, O. Uzun, and S. Vartivarian.** 1998. Predictors of adverse outcome in cancer patients with candidemia. *Am. J. Med.* **104:**238–245.
3. **Anastos, K., L. A. Kalish, H. Palacio, C. A. Benson, R. Delapenha, K. Chirgwin, L. Stonis, and E. E. Telzak.** 1999. Prevalence of and risk factors for tuberculin positivity and skin test anergy in HIV-1-infected and uninfected at-risk women. Women's Interagency HIV Study (WIHS). *J. Acquir. Immune Defic. Syndr.* **21:**141–147.
4. **Arnow, P. M., E. M. Quimosing, and M. Beach.** 1993. Consequences of intravascular catheter sepsis. *Clin. Infect. Dis.* **16:**778–784.
5. **Arrowood, J. R., and M. S. Hayney.** 2002. Immunization recommendations for adults with cancer. *Ann. Pharmacother.* **36:**1219–1229.
6. **Band, J. D., D. W. Fraser, and G. Ajello.** 1984. Prevention of *Hemophilus influenzae* type b disease. *JAMA* **251:**2381–2386.
7. **Bardare, M., L. Armenio, L. Businco, G. Cavagni, M. De Martino, M. Duse, M. Masi, V. Monafo, M. C. Pietrogrande, P. Tovo, et al.** 1990. Screening for delayed-hypersensitivity in Italian children: multicentric study by multitest skin testing. *Eur. J. Pediatr.* **150:**37–39.
8. **Bernstein, J. M., H. S. Faden, and P. L. Ogra.** 1991. Nasopharyngeal colonization by nontypeable *Hemophilus influenzae* in children: the effect of serum bactericidal antibody. *Otolaryngol. Head Neck Surg.* **105:**406–410.
9. **Black, S., H. Shinefield, B. Fireman, E. Lewis, P. Ray, J. R. Hansen, L. Elvin, K. M. Ensor, J. Hackell, G. Siber, F. Malinoski, D. Madore, I. Chang, R. Kohberger, W. Watson, R. Austrian, and K. Edwards for the Northern California Kaiser Permanente Vaccine Study Center Group.** 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. *Pediatr. Infect. Dis. J.* **19:**187–195.
10. **Bluestein, D., C. Rutledge, and L. Lumsden.** 1991. Predicting the occurrence of antibiotic-induced candidal vaginitis (AICV). *Fam. Pract. Res. J.* **11:**319–326.
11. **Boken, D. J., S. A. Chartrand, R. V. Goering, R. Kruger, and C. J. Harrison.** 1995. Colonization with penicillin-resistant *Streptococcus pneumoniae* in a child-care center. *Pediatr. Infect. Dis. J.* **14:**879–884.
12. **Boken, D. J., S. A. Chartrand, E. S. Moland, and R. V. Goering.** 1996. Colonization with penicillin-nonsusceptible *Streptococcus pneumoniae* in urban and rural child-care centers. *Pediatr. Infect. Dis. J.* **15:**667–672.
13. **Bolan, G., C. V. Broome, R. R. Facklam, B. D. Plikaytis, D. W. Fraser, and W. F. Schlech III.** 1986. Pneumococcal vaccine efficacy in selected populations in the United States. *Ann. Intern. Med.* **104:**1–6.
14. **Buhl, R.** 2006. Local oropharyngeal side effects of inhaled corticosteroids in patients with asthma. *Allergy* **61:** 518–526.
15. **Caesar-TonThat, T. C., and J. E. Cutler.** 1997. A monoclonal antibody to *Candida albicans* enhances mouse neutrophil candidacidal activity. *Infect. Immun.* **65:** 5354–5357.
16. **Chang, H. M., T. Y. Shann, K. T. Mok, W. Y. Lui, F. K. P'Eng, and H. Y. Lin.** 1989. Multitest skin testing values of cell-mediated immunity among healthy individuals in Taiwan. *Zhonghua Yi Xue Za Zhi* (Taipei) **44:**81–88.
17. **Chokephaibulkit, K., W. Phongsamart, N. Vanprapar, T. Chotpitayasunondh, and S. Chearskul.** 2004. Catch-up vaccination against *Haemophilus influenzae* type b in human immunodeficiency virus-infected Thai children older than 2 years old. *Vaccine* **22:**2018–2022.
18. **Collin, B., C. J. Clancy, and M. H. Nguyen.** 1999. Antifungal resistance in non-*albicans Candida* species. *Drug Resist. Updat.* **2:**9–14.
19. **Cormack, B. P., N. Ghori, and S. Falkow.** 1999. An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285:**578–582.
20. **Creditor, M. C., E. C. Smith, J. B. Gallai, M. Baumann, and K. E. Nelson.** 1988. Tuberculosis, tuberculin reactivity, and delayed cutaneous hypersensitivity in nursing home residents. *J. Gerontol.* **43:**M97–M100.
21. **Cutler, J. E.** 2005. Defining criteria for anti-mannan antibodies to protect against candidiasis. *Curr. Mol. Med.* **5:**383–392.
22. **DeGregorio, M. W., W. M. Lee, and C. A. Ries.** 1982. *Candida* infections in patients with acute leukemia: ineffectiveness of nystatin prophylaxis and relationship between oropharyngeal and systemic candidiasis. *Cancer* **50:**2780–2784.
23. **De Lencastre, H., K. G. Kristinsson, A. Brito-Avo, I. S. Sanches, R. Sa-Leao, J. Saldanha, E. Sigvaldadottir, S. Karlsson, D. Oliveira, R. Mato, M. Aires de Sousa, and A. Tomasz.** 1999. Carriage of respiratory tract pathogens and molecular epidemiology of *Streptococcus pneumoniae* colonization in healthy children attending day care centers in Lisbon, Portugal. *Microb. Drug Resist.* **5:**19–29.
24. **Derendorf, H., R. Nave, A. Drollmann, F. Cerasoli, and W. Wurst.** 2006. Relevance of pharmacokinetics and pharmacodynamics of inhaled corticosteroids to asthma. *Eur. Respir. J.* **28:**1042–1050.
25. **De Sousa dos Santos, S., M. H. Lopes, V. Simonsen, and H. H. Caiaffa Filho.** 2004. *Haemophilus influenzae* type b immunization in adults infected with the human immunodeficiency virus. *AIDS Res. Hum. Retroviruses* **20:**493–496.
26. **Dockrell, D. H., G. A. Poland, J. M. Steckelberg, P. C. Wollan, S. R. Strickland, and C. Pomeroy.** 1999. Immunogenicity of three *Haemophilus influenzae* type b protein conjugate vaccines in HIV seropositive adults and analysis of predictors of vaccine response. *Vaccine* **17:**2779–2785.
27. **Dongari-Bagtzoglou, A., and P. L. Fidel, Jr.** 2005. The host cytokine responses and protective immunity in oropharyngeal candidiasis. *J. Dent. Res.* **84:**966–977.
28. **Duerr, A., C. M. Heilig, S. F. Meikle, S. Cu-Uvin, R. S. Klein, A. Rompalo, and J. D. Sobel.** 2003. Incident and persistent vulvovaginal candidiasis among human immunodeficiency virus-infected women: risk factors and severity. *Obstet. Gynecol.* **101:**548–556.
29. **Edwards, J. E., Jr., C. L. Mayer, S. G. Filler, E. Wadsworth, and R. A. Calderone.** 1992. Cell extracts of *Candida albicans* block adherence of the organisms to endothelial cells. *Infect. Immun.* **60:**3087–3091.
30. **Eskola, J., H. Kayhty, A. K. Takala, H. Peltola, P. R. Ronnberg, E. Kela, E. Pekkanen, P. H. McVerry, and P. H. Makela.** 1990. A randomized, prospective field trial of a conjugate vaccine in the protection of infants and young children against invasive *Haemophilus influenzae* type b disease. *N. Engl. J. Med.* **323:**1381–1387.
31. **Feldman, S., F. Gigliotti, J. L. Shenep, P. K. Roberson, and L. Lott.** 1990. Risk of *Haemophilus influenzae* type b disease in children with cancer and response of immunocompromised leukemic children to a conjugate vaccine. *J. Infect. Dis.* **161:**926–931.
32. **Fidel, P. L., Jr.** 2007. History and update on host defense against vaginal candidiasis. *Am. J. Reprod. Immunol.* **57:**2–12.
33. **Fidel, P. L., Jr., M. Barousse, V. Lounev, T. Espinosa, R. R. Chesson, and K. Dunlap.** 2003. Local immune responsiveness following intravaginal challenge with *Candida* antigen in adult women at different stages of the menstrual cycle. *Med. Mycol.* **41:**97–109.
34. **Foxman, B., R. Barlow, H. D'Arcy, B. Gillespie, and J. D. Sobel.** 2000. *Candida* vaginitis: self-reported incidence and associated costs. *Sex. Transm. Dis.* **27:**230–235.

35. **Foxman, B., J. V. Marsh, B. Gillespie, and J. D. Sobel.** 1998. Frequency and response to vaginal symptoms among white and African American women: results of a random digit dialing survey. *J. Womens Health* **7:**1167–1174.
36. **Fraser, V. J., M. Jones, J. Dunkel, S. Storfer, G. Medoff, and W. C. Dunagan.** 1992. Candidemia in a tertiary care hospital: epidemiology, risk factors, and predictors of mortality. *Clin. Infect. Dis.* **15:**414–421.
37. **Frieman, M. B., J. M. McCaffery, and B. P. Cormack.** 2002. Modular domain structure in the *Candida glabrata* adhesin Epa1p, a β1,6 glucan-cross-linked cell wall protein. *Mol. Microbiol.* **46:**479–492.
38. **Gable, C. B., S. S. Holzer, L. Engelhart, R. B. Friedman, F. Smeltz, D. Schroeder, and K. Baum.** 1990. Pneumococcal vaccine. Efficacy and associated cost savings. *JAMA* **264:**2910–2915.
39. **Giraldo, P. C., O. Babula, A. K. Goncalves, I. M. Linhares, R. L. Amaral, W. J. Ledger, and S. S. Witkin.** 2007. Mannose-binding lectin gene polymorphism, vulvovaginal candidiasis, and bacterial vaginosis. *Obstet. Gynecol.* **109:**1123–1128.
40. **Gray, B. M., G. M. Converse III, and H. C. Dillon, Jr.** 1980. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J. Infect. Dis.* **142:**923–933.
41. **Gray, B. M., and H. C. Dillon, Jr.** 1988. Epidemiological studies of *Streptococcus pneumoniae* in infants: antibody to types 3, 6, 14, and 23 in the first two years of life. *J. Infect. Dis.* **158:**948–955.
42. **Greenwood, B.** 2005. Interpreting vaccine efficacy. *Clin. Infect. Dis.* **40:**1519–1520.
43. **Gudlaugsson, O., S. Gillespie, K. Lee, J. Vande Berg, J. Hu, S. Messer, L. Herwaldt, M. Pfaller, and D. Diekema.** 2003. Attributable mortality of nosocomial candidemia, revisited. *Clin. Infect. Dis.* **37:**1172–1177.
44. **Guerrero, M., S. Kruger, A. Saitoh, F. Sorvillo, K. J. Cheng, C. French, and G. Beall.** 1999. Pneumonia in HIV-infected patients: a case-control survey of factors involved in risk and prevention. *AIDS* **13:**1971–1975.
45. **Hajjeh, R. A., A. N. Sofair, L. H. Harrison, G. M. Lyon, B. A. Arthington-Skaggs, S. A. Mirza, M. Phelan, J. Morgan, W. Lee-Yang, M. A. Ciblak, L. E. Benjamin, L. T. Sanza, S. Huie, S. F. Yeo, M. E. Brandt, and D. W. Warnock.** 2004. Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J. Clin. Microbiol.* **42:**1519–1527.
46. **Han, Y., and J. E. Cutler.** 1995. Antibody response that protects against disseminated candidiasis. *Infect. Immun.* **63:**2714–2719.
47. **Han, Y., T. Kanbe, R. Cherniak, and J. E. Cutler.** 1997. Biochemical characterization of *Candida albicans* epitopes that can elicit protective and nonprotective antibodies. *Infect. Immun.* **65:**4100–4107.
48. **Han, Y., R. P. Morrison, and J. E. Cutler.** 1998. A vaccine and monoclonal antibodies that enhance mouse resistance to *Candida albicans* vaginal infection. *Infect. Immun.* **66:**5771–5776.
49. **Han, Y., M. H. Riesselman, and J. E. Cutler.** 2000. Protection against candidiasis by an immunoglobulin G3 (IgG3) monoclonal antibody specific for the same mannotriose as an IgM protective antibody. *Infect. Immun.* **68:**1649–1654.
50. **Han, Y., M. A. Ulrich, and J. E. Cutler.** 1999. *Candida albicans* mannan extract-protein conjugates induce a protective immune response against experimental candidiasis. *J. Infect. Dis.* **179:**1477–1484.
51. **Haroon Parupia, M. F., and R. Dhanireddy.** 2001. Association of postnatal dexamethasone use and fungal sepsis in the development of severe retinopathy of prematurity and progression to laser therapy in extremely low-birth-weight infants. *J. Perinatol.* **21:**242–247.
52. **Heidenreich, S., T. Kubis, M. Schmidt, and W. Fegeler.** 1994. Glucocorticoid-induced alterations of monocyte defense mechanisms against *Candida albicans*. *Cell. Immunol.* **157:**320–327.
53. **Hodgetts, S., L. Nooney, R. Al-Akeel, A. Curry, S. Awad, R. Matthews, and J. Burnie.** 2008. Efungumab and caspofungin: pre-clinical data supporting synergy. *J. Antimicrob. Chemother.* **61:**1132–1139.
54. **Hope, W., A. Morton, and D. P. Eisen.** 2002. Increase in prevalence of nosocomial non-*Candida albicans* candidaemia and the association of *Candida krusei* with fluconazole use. *J. Hosp. Infect.* **50:**56–65.
55. **Hoyer, L. L.** 2001. The ALS gene family of *Candida albicans*. *Trends Microbiol.* **9:**176–180.
56. **Hoyer, L. L., R. Fundyga, J. E. Hecht, J. C. Kapteyn, F. M. Klis, and J. Arnold.** 2001. Characterization of agglutinin-like sequence genes from non-*albicans Candida* and phylogenetic analysis of the ALS family. *Genetics* **157:**1555–1567.
57. **Ibrahim, A. S., B. J. Spellberg, V. Avanesian, Y. Fu, and J. E. J. Edwards.** 2006. The anti-*Candida* rAls1p-N vaccine is broadly active against disseminated candidiasis. *Infect. Immun.* **74:**3039–3041.
58. **Ibrahim, A. S., B. J. Spellberg, V. Avenissian, Y. Fu, S. G. Filler, and J. E. Edwards, Jr.** 2005. Vaccination with recombinant N-terminal domain of Als1p improves survival during murine disseminated candidiasis by enhancing cell-mediated, not humoral, immunity. *Infect. Immun.* **73:**999–1005.
59. **Kao, A. S., M. E. Brandt, W. R. Pruitt, L. A. Conn, B. A. Perkins, D. S. Stephens, W. S. Baughman, A. L. Reingold, G. A. Rothrock, M. A. Pfaller, R. W. Pinner, and R. A. Hajjeh.** 1999. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. *Clin. Infect. Dis.* **29:**1164–1170.
60. **Karabinis, A., C. Hill, B. Leclercq, C. Tancrede, D. Baume, and A. Andremont.** 1988. Risk factors for candidemia in cancer patients: a case-control study. *J. Clin. Microbiol.* **26:**429–432.
61. **Karlowsky, J. A., G. G. Zhanel, K. A. Klym, D. J. Hoban, and A. M. Kabani.** 1997. Candidemia in a Canadian tertiary care hospital from 1976 to 1996. *Diagn. Microbiol. Infect. Dis.* **29:**5–9.
62. **Kelly, S. L., D. C. Lamb, D. E. Kelly, N. J. Manning, J. Loeffler, H. Hebart, U. Schumacher, and H. Einsele.** 1997. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol Δ5,6-desaturation. *FEBS Lett.* **400:**80–82.
63. **Kemper, C. A., R. Haubrich, I. Frank, G. Dubin, C. Buscarino, J. A. McCutchan, and S. C. Deresinski.** 2003. Safety and immunogenicity of hepatitis A vaccine in human immunodeficiency virus-infected patients: a double-blind, randomized, placebo-controlled trial. *J. Infect. Dis.* **187:**1327–1331.
64. **Kimura, L. H., and N. N. Pearsall.** 1978. Adherence of *Candida albicans* to human buccal epithelial cells. *Infect. Immun.* **21:**64–68.
65. **King, J. C., Jr., P. E. Vink, J. J. Farley, M. Parks, M. Smilie, D. Madore, R. Lichenstein, and F. Malinoski.** 1996. Comparison of the safety and immunogenicity of a pneumococcal conjugate with a licensed polysaccharide vaccine in human immunodeficiency virus and non-human immunodeficiency virus-infected children. *Pediatr. Infect. Dis. J.* **15:**192–196.
66. **Klotz, S. A., and R. L. Smith.** 1995. Gelatin fragments block adherence of *Candida albicans* to extracellular matrix proteins. *Microbiology* **141**(Pt. 10)**:**2681–2684.

67. **Klugman, K. P., S. A. Madhi, R. E. Huebner, R. Kohberger, N. Mbelle, and N. Pierce.** 2003. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. *N. Engl. J. Med.* **349:** 1341–1348.
68. **Koivula, I., M. Sten, M. Leinonen, and P. H. Makela.** 1997. Clinical efficacy of pneumococcal vaccine in the elderly: a randomized, single-blind population-based trial. *Am. J. Med.* **103:**281–290.
69. **Kossoff, E. H., E. S. Buescher, and M. G. Karlowicz.** 1998. Candidemia in a neonatal intensive care unit: trends during fifteen years and clinical features of 111 cases. *Pediatr. Infect. Dis. J.* **17:**504–508.
70. **Kroon, F. P., J. T. van Dissel, E. Ravensbergen, P. H. Nibbering, and R. van Furth.** 1999. Impaired antibody response after immunization of HIV-infected individuals with the polysaccharide vaccine against *Salmonella typhi* (Typhim-Vi). *Vaccine* **17:**2941–2945.
71. **Kullberg, B. J., and A. M. Oude Lashof.** 2002. Epidemiology of opportunistic invasive mycoses. *Eur. J. Med. Res.* **7:**183–191.
72. **Kurtti, P., R. Isoaho, L. von Hertzen, T. Keistinen, S. L. Kivela, and M. Leinonen.** 1997. Influence of age, gender and smoking on *Streptococcus pneumoniae, Haemophilus influenzae and Moraxella (Branhamella) catarrhalis* antibody titres in an elderly population. *Scand. J. Infect. Dis.* **29:** 485–489.
73. **Lark, R. L., C. Chenoweth, S. Saint, J. K. Zemencuk, B. A. Lipsky, and J. J. Plorde.** 2000. Four year prospective evaluation of nosocomial bacteremia: epidemiology, microbiology, and patient outcome. *Diagn. Microbiol. Infect. Dis.* **38:**131–140.
74. **Launay, O., O. Lortholary, C. Bouges-Michel, B. Jarrousse, M. Bentata, and L. Guillevin.** 1998. Candidemia: a nosocomial complication in adults with late-stage AIDS. *Clin. Infect. Dis.* **26:**1134–1141.
75. **Leino, T., K. Auranen, P. H. Makela, H. Kayhty, M. Ramsay, M. Slack, and A. K. Takala.** 2002. *Haemophilus influenzae* type b and cross-reactive antigens in natural Hib infection dynamics; modelling in two populations. *Epidemiol. Infect.* **129:**73–83.
76. **Leung, T. F., C. K. Li, E. C. Hung, P. K. Chan, C. W. Mo, R. P. Wong, and K. W. Chik.** 2004. Immunogenicity of a two-dose regime of varicella vaccine in children with cancers. *Eur. J. Haematol.* **72:**353–357.
77. **Levin, M. J., A. A. Gershon, A. Weinberg, S. Blanchard, B. Nowak, P. Palumbo, and C. Y. Chan.** 2001. Immunization of HIV-infected children with varicella vaccine. *J. Pediatr.* **139:**305–310.
78. **Lifshitz, S., R. Dagan, M. Shani-Sekler, N. Grossman, G. Fleminger, M. Friger, and Y. M. Nebenzahl.** 2002. Age-dependent preference in human antibody responses to *Streptococcus pneumoniae* polypeptide antigens. *Clin. Exp. Immunol.* **127:**344–353.
79. **Lin, L., A. S. Ibrahim, V. Avanesian, J. E. J. Edwards, Y. Fu, B. Baquir, R. Taub, and B. Spellberg.** 2008. Vaccine immunogenicity and efficacy vary considerably by diluent used for aluminum hydroxide adjuvant. *Clin. Vaccine Immunol.* **15:**582–584.
80. **Lin, L., A. S. Ibrahim, X. Xu, J. M. Farber, V. Avanesian, B. Baquir, Y. Fu, S. W. French, J. E. Edwards, Jr., and B. Spellberg.** 2009. Th1-Th17 cells mediate protective adaptive immunity against *Staphylococcus aureus* and *Candida albicans* infection in mice. *PLoS Pathog.* **5:**e1000703.
81. **Loda, F. A., A. M. Collier, W. P. Glezen, K. Strangert, W. A. Clyde, Jr., and F. W. Denny.** 1975. Occurrence of *Diplococcus pneumoniae* in the upper respiratory tract of children. *J. Pediatr.* **87:**1087–1093.
82. **Luzzati, R., G. Amalfitano, L. Lazzarini, F. Soldani, S. Bellino, M. Solbiati, M. C. Danzi, S. Vento, G. Todeschini, C. Vivenza, and E. Concia.** 2000. Nosocomial candidemia in non-neutropenic patients at an Italian tertiary care hospital. *Eur. J. Clin. Microbiol. Infect. Dis.* **19:**602–607.
83. **Madhi, S. A., L. Kuwanda, C. Cutland, and K. P. Klugman.** 2005. The impact of a 9-valent pneumococcal conjugate vaccine on the public health burden of pneumonia in HIV-infected and -uninfected children. *Clin. Infect. Dis.* **40:**1511–1518.
84. **Madhi, S. A., K. Petersen, M. Khoosal, R. E. Huebner, N. Mbelle, R. Mothupi, H. Saloojee, H. Crewe-Brown, and K. P. Klugman.** 2002. Reduced effectiveness of *Haemophilus influenzae* type b conjugate vaccine in children with a high prevalence of human immunodeficiency virus type 1 infection. *Pediatr. Infect. Dis. J.* **21:**315–321.
85. **Madore, D. V.** 1996. Impact of immunization on *Haemophilus influenzae* type b disease. *Infect. Agents Dis.* **5:**8–20.
86. **Malazy, O. T., M. Shariat, R. Heshmat, F. Majlesi, M. Alimohammadian, N. K. Tabari, and B. Larijani.** 2007. Vulvovaginal candidiasis and its related factors in diabetic women. *Taiwan J. Obstet. Gynecol.* **46:**399–404.
87. **Marr, K. A., K. Seidel, T. C. White, and R. A. Bowden.** 2000. Candidemia in allogeneic blood and marrow transplant recipients: evolution of risk factors after the adoption of prophylactic fluconazole. *J. Infect. Dis.* **181:**309–316.
88. **Martino, P., C. Girmenia, A. Micozzi, R. Raccah, G. Gentile, M. Venditti, and F. Mandelli.** 1993. Fungemia in patients with leukemia. *Am. J. Med. Sci.* **306:**225–232.
89. **Matthews, R., S. Hodgetts, and J. Burnie.** 1995. Preliminary assessment of a human recombinant antibody fragment to hsp90 in murine invasive candidiasis. *J. Infect. Dis.* **171:**1668–1671.
90. **Matthews, R. C., J. P. Burnie, D. Howat, T. Rowland, and F. Walton.** 1991. Autoantibody to heat-shock protein 90 can mediate protection against systemic candidosis. *Immunology* **74:**20–24.
91. **Matthews, R. C., J. P. Burnie, and S. Tabaqchali.** 1987. Isolation of immunodominant antigens from sera of patients with systemic candidiasis and characterization of serological response to *Candida albicans*. *J. Clin. Microbiol.* **25:**230–237.
92. **Matthews, R. C., G. Rigg, S. Hodgetts, T. Carter, C. Chapman, C. Gregory, C. Illidge, and J. Burnie.** 2003. Preclinical assessment of the efficacy of Mycograb, a human recombinant antibody against fungal HSP90. *Antimicrob. Agents Chemother.* **47:**2208–2216.
93. **Mayer, C. L., S. G. Filler, and J. E. Edwards, Jr.** 1992. *Candida albicans* adherence to endothelial cells. *Microvasc. Res.* **43:**218–226.
94. **Meunier, F., M. Aoun, and N. Bitar.** 1992. Candidemia in immunocompromised patients. *Clin. Infect. Dis.* **14**(Suppl. 1)**:**S120–S125.
95. **Miller, L. G., R. A. Hajjeh, and J. E. Edwards, Jr.** 2001. Estimating the cost of nosocomial candidemia in the United States. *Clin. Infect. Dis.* **32:**1110.
96. **Muller, F. M., M. Weig, J. Peter, and T. J. Walsh.** 2000. Azole cross-resistance to ketoconazole, fluconazole, itraconazole and voriconazole in clinical *Candida albicans* isolates from HIV-infected children with oropharyngeal candidosis. *J. Antimicrob. Chemother.* **46:**338–340.
97. **Murphy, E. L., Y. Wu, H. E. Ownby, J. W. Smith, R. K. Ruedy, R. A. Thomson, D. I. Ameti, D. J. Wright, and G. J. Nemo.** 2001. Delayed hypersensitivity skin testing to mumps and *Candida albicans* antigens is normal in middle-aged HTLV-I- and-II-infected U.S. cohorts. *AIDS Res. Hum. Retrovir.* **17:**1273–1277.

98. **Musher, D. M., J. E. Groover, M. R. Reichler, F. X. Riedo, B. Schwartz, D. A. Watson, R. E. Baughn, and R. F. Breiman.** 1997. Emergence of antibody to capsular polysaccharides of *Streptococcus pneumoniae* during outbreaks of pneumonia: association with nasopharyngeal colonization. *Clin. Infect. Dis.* **24:**441–446.
99. **Musher, D. M., J. E. Groover, J. M. Rowland, D. A. Watson, J. B. Struewing, R. E. Baughn, and M. A. Mufson.** 1993. Antibody to capsular polysaccharides of *Streptococcus pneumoniae*: prevalence, persistence, and response to revaccination. *Clin. Infect. Dis.* **17:**66–73.
100. **Musher, D. M., D. A. Watson, and R. E. Baughn.** 1990. Does naturally acquired IgG antibody to cell wall polysaccharide protect human subjects against pneumococcal infection? *J. Infect. Dis.* **161:**736–740.
101. **Netea, M. G., N. A. Gow, C. A. Munro, S. Bates, C. Collins, G. Ferwerda, R. P. Hobson, G. Bertram, H. B. Hughes, T. Jansen, L. Jacobs, E. T. Buurman, K. Gijzen, D. L. Williams, R. Torensma, A. McKinnon, D. M. MacCallum, F. C. Odds, J. W. Van der Meer, A. J. Brown, and B. J. Kullberg.** 2006. Immune sensing of *Candida albicans* requires cooperative recognition of mannans and glucans by lectin and Toll-like receptors. *J. Clin. Investig.* **116:**1642–1650.
102. **Nolla-Salas, J., A. Sitges-Serra, C. Leon-Gil, J. Martinez-Gonzalez, M. A. Leon-Regidor, P. Ibanez-Lucia, and J. M. Torres-Rodriguez.** 1997. Candidemia in nonneutropenic critically ill patients: analysis of prognostic factors and assessment of systemic antifungal therapy. Study Group of Fungal Infection in the ICU. *Intensive Care Med.* **23:**23–30.
103. **Nordoy, T., I. S. Aaberge, A. Husebekk, H. H. Samdal, S. Steinert, H. Melby, and A. Kolstad.** 2002. Cancer patients undergoing chemotherapy show adequate serological response to vaccinations against influenza virus and *Streptococcus pneumoniae*. *Med. Oncol.* **19:**71–78.
104. **Nucci, M., W. Pulcheri, N. Spector, A. P. Bueno, P. C. Bacha, M. J. Caiuby, A. Derossi, R. Costa, J. C. Morais, and H. P. de Oliveira.** 1995. Fungal infections in neutropenic patients. A 8-year prospective study. *Rev. Inst. Med. Trop. São Paulo* **37:**397–406.
105. **Ohri, L. K., J. M. Manley, A. Chatterjee, and N. E. Cornish.** 2004. Pediatric case series evaluating a standardized *Candida albicans* skin test product. *Ann. Pharmacother.* **38:**973–977.
106. **Opal, S. M., G. E. Garber, S. P. LaRosa, D. G. Maki, R. C. Freebairn, G. T. Kinasewitz, J. F. Dhainaut, S. B. Yan, M. D. Williams, D. E. Graham, D. R. Nelson, H. Levy, and G. R. Bernard.** 2003. Systemic host responses in severe sepsis analyzed by causative microorganism and treatment effects of drotrecogin alfa (activated). *Clin. Infect. Dis.* **37:**50–58.
107. **Pachl, J., P. Svoboda, F. Jacobs, K. Vandewoude, B. van der Hoven, P. Spronk, G. Masterson, M. Malbrain, M. Aoun, J. Garbino, J. Takala, L. Drgona, J. Burnie, and R. Matthews.** 2006. A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. *Clin. Infect. Dis.* **42:**1404–1413.
108. **Pagano, L., A. Antinori, A. Ammassari, L. Mele, A. Nosari, L. Melillo, B. Martino, M. Sanguinetti, F. Equitani, F. Nobile, M. Carotenuto, E. Morra, G. Morace, and G. Leone.** 1999. Retrospective study of candidemia in patients with hematological malignancies. Clinical features, risk factors and outcome of 76 episodes. *Eur. J. Haematol.* **63:**77–85.
109. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, and J. D. Sobel.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.
110. **Pappas, P. G., J. H. Rex, J. Lee, R. J. Hamill, R. A. Larsen, W. Powderly, C. A. Kauffman, N. Hyslop, J. E. Mangino, S. Chapman, H. W. Horowitz, J. E. Edwards, and W. E. Dismukes.** 2003. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin. Infect. Dis.* **37:**634–643.
111. **Parveen, N., A. A. Munir, I. Din, and R. Majeed.** 2008. Frequency of vaginal candidiasis in pregnant women attending routine antenatal clinic. *J. Coll. Physicians Surg. Pak.* **18:**154–157.
112. **Paul, W. E. (ed.).** 2003. *Fundamental Immunology*, 5th ed. Lippincott Williams & Wilkins, New York, NY.
113. **Perlroth, J., B. Choi, and B. Spellberg.** 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med. Mycol.* **45:**321–346.
114. **Peter, C. G.** 1998. Responses of children immunized with capsular polysaccharide of *Hemophilus influenzae* type b, by David H. Smith, MD, et al, Pediatrics, 1973;52:637–644; and *Haemophilus influenzae* type b capsular polysaccharide vaccine in children: a double-blind field study of 100 000 vaccinees 3 months to 5 years of age in Finland, by Heikki Peltola, MD et al, Pediatrics, 1977;60:730–737. *Pediatrics* **102:**252–254.
115. **Pfaller, M. A.** 1995. Epidemiology of candidiasis. *J. Hosp. Infect.* **30**(Suppl.)**:**329–338.
116. **Pirotta, M. V., J. M. Gunn, and P. Chondros.** 2003. "Not thrush again!" Women's experience of post-antibiotic vulvovaginitis. *Med. J. Aust.* **179:**43–46.
117. **Powderly, W. G., K. H. Mayer, and J. R. Perfect.** 1999. Diagnosis and treatment of oropharyngeal candidiasis in patients infected with HIV: a critical reassessment. *AIDS Res. Hum. Retrovir.* **15:**1405–1412.
118. **Rachini, A., D. Pietrella, P. Lupo, A. Torosantucci, P. Chiani, C. Bromuro, C. Proietti, F. Bistoni, A. Cassone, and A. Vecchiarelli.** 2007. An anti-beta-glucan monoclonal antibody inhibits growth and capsule formation of *Cryptococcus neoformans* in vitro and exerts therapeutic, anticryptococcal activity in vivo. *Infect. Immun.* **75:**5085–5094.
119. **Rangel-Frausto, M. S., T. Wiblin, H. M. Blumberg, L. Saiman, J. Patterson, M. Rinaldi, M. Pfaller, J. E. J. Edwards, W. Jarvis, J. Dawson, and R. P. Wenzel.** 1999. National Epidemiology of Mycoses Survey (NEMIS): variations in rates of bloodstream infections due to *Candida* species in seven surgical intensive care units and six neonatal intensive care units. *Clin. Infect. Dis.* **29:**253–258.
120. **Raymond, J., I. Le Thomas, F. Moulin, A. Commeau, D. Gendrel, and P. Berche.** 2000. Sequential colonization by *Streptococcus pneumoniae* of healthy children living in an orphanage. *J. Infect. Dis.* **181:**1983–1988.
121. **Read, J. S., C. E. Frasch, K. Rich, G. A. Fitzgerald, J. D. Clemens, J. Pitt, S. I. Pelton, I. C. Hanson, E. Handelsman, C. Diaz, and M. G. Fowler for the Women and Infants Transmission Study Group.** 1998. The immunogenicity of *Haemophilus influenzae* type b conjugate vaccines in children born to human immunodeficiency virus-infected women. *Pediatr. Infect. Dis. J.* **17:**391–397.
122. **Redding, S. W., R. C. Zellars, W. R. Kirkpatrick, R. K. McAtee, M. A. Caceres, A. W. Fothergill, J. L. Lopez-Ribot, C. W. Bailey, M. G. Rinaldi, and T. F. Patterson.** 1999. Epidemiology of oropharyngeal *Candida* colonization and infection in patients receiving radiation for head and neck cancer. *J. Clin. Microbiol.* **37:**3896–3900.

123. **Rennert, G., H. S. Rennert, S. Pitlik, R. Finkelstein, and R. Kitzes-Cohen.** 2000. Epidemiology of candidemia—a nationwide survey in Israel. *Infection* **28:**26–29.
124. **Rich, R. R., T. A. Fleisher, W. T. Shearer, B. L. Kotzin, and H. W. J. Schroeder (ed.).** 2001. *Clinical Immunology: Principles and Practice*, 2nd ed. Mosby, New York, NY.
125. **Richet, H. M., A. Andremont, C. Tancrede, J. L. Pico, and W. R. Jarvis.** 1991. Risk factors for candidemia in patients with acute lymphocytic leukemia. *Rev. Infect. Dis.* **13:**211–215.
126. **Rodriguez-Barradas, M. C., R. A. Tharapel, J. E. Groover, K. P. Giron, C. E. Lacke, E. D. Houston, R. J. Hamill, M. C. Steinhoff, and D. M. Musher.** 1997. Colonization by *Streptococcus pneumoniae* among human immunodeficiency virus-infected adults: prevalence of antibiotic resistance, impact of immunization, and characterization by polymerase chain reaction with BOX primers of isolates from persistent *S. pneumoniae* carriers. *J. Infect. Dis.* **175:**590–597.
127. **Rutstein, R. M., B. J. Rudy, and A. Cnaan.** 1996. Response of human immunodeficiency virus-exposed and -infected infants to *Haemophilus influenzae* type b conjugate vaccine. *Arch. Pediatr. Adolesc. Med.* **150:**838–841.
128. **Saiman, L., E. Ludington, M. Pfaller, S. Rangel-Frausto, R. T. Wiblin, J. Dawson, H. M. Blumberg, J. E. Patterson, M. Rinaldi, J. E. Edwards, R. P. Wenzel, and W. Jarvis for The National Epidemiology of Mycosis Survey Study Group.** 2000. Risk factors for candidemia in Neonatal Intensive Care Unit patients. *Pediatr. Infect. Dis. J.* **19:**319–324.
129. **Schuman, P., S. E. Ohmit, J. D. Sobel, K. H. Mayer, V. Greene, A. Rompalo, and R. S. Klein for the HIV Epidemiology Research Study (HERS) Group.** 1998. Oral lesions among women living with or at risk for HIV infection. *Am. J. Med.* **104:**559–564.
130. **Schuman, P., J. D. Sobel, S. E. Ohmit, K. H. Mayer, C. C. Carpenter, A. Rompalo, A. Duerr, D. K. Smith, D. Warren, and R. S. Klein for the HIV Epidemiology Research Study (HERS) Group.** 1998. Mucosal candidal colonization and candidiasis in women with or at risk for human immunodeficiency virus infection. *Clin. Infect. Dis.* **27:**1161–1167.
131. **Shapiro, E. D., A. T. Berg, R. Austrian, D. Schroeder, V. Parcells, A. Margolis, R. K. Adair, and J. D. Clemens.** 1991. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. *N. Engl. J. Med.* **325:**1453–1460.
132. **Shapiro, E. D., and J. D. Clemens.** 1984. A controlled evaluation of the protective efficacy of pneumococcal vaccine for patients at high risk of serious pneumococcal infections. *Ann. Intern. Med.* **101:**325–330.
133. **Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu, A. S. Ibrahim, S. G. Filler, M. Zhang, A. J. Waring, and J. E. Edwards, Jr.** 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. *J. Biol. Chem.* **279:**30480–30489.
134. **Sinsalo, M., J. Aittoniemi, H. Kayhty, and J. Vilpo.** 2002. *Haemophilus influenzae* type b (Hib) antibody concentrations and vaccination responses in patients with chronic lymphocytic leukaemia: predicting factors for response. *Leuk. Lymphoma* **43:**1967–1969.
135. **Smit, P., D. Oberholzer, S. Hayden-Smith, H. J. Koornhof, and M. R. Hilleman.** 1977. Protective efficacy of pneumococcal polysaccharide vaccines. *JAMA* **238:** 2613–2616.
136. **Sobel, J. D.** 2007. Vulvovaginal candidosis. *Lancet* **369:**1961–1971.
137. **Somjee, S., S. Pai, P. Parikh, S. Banavali, R. Kelkar, and S. Advani.** 2002. Passive active prophylaxis against hepatitis B in children with acute lymphoblastic leukemia. *Leuk. Res.* **26:**989–992.
138. **Spellberg, B., S. G. Filler, and J. E. Edwards Jr.** 2006. Current treatment strategies for disseminated candidiasis. *Clin. Infect. Dis.* **42:**244–251.
139. **Spellberg, B., A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2005. Mice with disseminated candidiasis die of progressive sepsis. *J. Infect. Dis.* **192:**336–343.
140. **Spellberg, B., A. S. Ibrahim, L. Lin, V. Avanesian, Y. Fu, P. Lipke, H. Otoo, T. Ho, and J. E. Edwards, Jr.** 2008. An antibody titer threshold predicts anti-candidal vaccine efficacy even though the mechanism of protection is induction of cell-mediated immunity. *J. Infect. Dis.* **197:**957–971.
141. **Spellberg, B. J., and J. E. Edwards, Jr.** 2002. The pathophysiology and treatment of *Candida* sepsis. *Curr. Infect. Dis. Rep.* **4:**387–399.
142. **Spellberg, B. J., A. S. Ibrahim, V. Avanesian, Y. Fu, C. Myers, Q. T. Phan, S. G. Filler, M. R. Yeaman, and J. E. Edwards, Jr.** 2006. Efficacy of the anti-*Candida* rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J. Infect. Dis.* **194:**256–260.
143. **Spellberg, B. J., A. S. Ibrahim, V. Avenissian, S. G. Filler, C. L. Myers, Y. Fu, and J. E. Edwards, Jr.** 2005. The anti-*Candida albicans* vaccine composed of the recombinant N terminus of Als1p reduces fungal burden and improves survival in both immunocompetent and immunocompromised mice. *Infect. Immun.* **73:** 6191–6193.
144. **Spellberg, B. J., D. Johnston, Q. T. Phan, J. E. Edwards, Jr., S. W. French, A. Ibrahim, and S. G. Filler.** 2003. Parenchymal organ, and not splenic, immunity correlates with host survival during disseminated candidiasis. *Infect. Immun.* **71:**5756–5764.
145. **Spinillo, A., E. Capuzzo, S. Acciano, A. De Santolo, and F. Zara.** 1999. Effect of antibiotic use on the prevalence of symptomatic vulvovaginal candidiasis. *Am. J. Obstet. Gynecol.* **180:**14–17.
146. **Steele, R. W., R. Warrier, P. J. Unkel, B. J. Foch, R. F. Howes, S. Shah, K. Williams, S. Moore, and S. J. Jue.** 1996. Colonization with antibiotic-resistant *Streptococcus pneumoniae* in children with sickle cell disease. *J. Pediatr.* **128:**531–535.
147. **Stockinger, B., and M. Veldhoen.** 2007. Differentiation and function of Th17 T cells. *Curr. Opin. Immunol.* **19:**281–286.
148. **Stone, H. H.** 1974. Studies in the pathogenesis, diagnosis, and treatment of *Candida* sepsis in children. *J. Pediatr. Surg.* **9:**127–133.
149. **Tasker, S. A., G. A. Schnepf, M. Lim, H. E. Caraviello, A. Armstrong, M. Bavaro, B. K. Agan, J. Delmar, N. Aronson, M. R. Wallace, and J. D. Grabenstein.** 2004. Unintended smallpox vaccination of HIV-1-infected individuals in the United States military. *Clin. Infect. Dis.* **38:**1320–1322.
150. **Tedaldi, E. M., R. K. Baker, A. C. Moorman, K. C. Wood, J. Fuhrer, R. E. McCabe, and S. D. Holmberg.** 2004. Hepatitis A and B vaccination practices for ambulatory patients infected with HIV. *Clin. Infect. Dis.* **38:**1478–1484.
151. **Torosantucci, A., C. Bromuro, P. Chiani, F. De Bernardis, F. Berti, C. Galli, F. Norelli, C. Bellucci, L. Polonelli, P. Costantino, R. Rappuoli, and A. Cassone.** 2005. A novel glyco-conjugate vaccine against fungal pathogens. *J. Exp. Med.* **202:**597–606.
152. **Tumbarello, M., E. Tacconelli, K. de Gaetano Donati, G. Morace, G. Fadda, and R. Cauda.** 1999. Candidemia in HIV-infected subjects. *Eur. J. Clin. Microbiol. Infect. Dis.* **18:**478–483.

153. **Uzun, O., and E. J. Anaissie.** 2000. Predictors of outcome in cancer patients with candidemia. *Ann. Oncol.* **11:**1517–1521.
154. **Uzun, O., S. Ascioglu, E. J. Anaissie, and J. H. Rex.** 2001. Risk factors and predictors of outcome in patients with cancer and breakthrough candidemia. *Clin. Infect. Dis.* **32:**1713–1717.
155. **Valentin, A., R. Le Guennec, E. Rodriguez, J. Reynes, M. Mallie, and J. M. Bastide.** 1996. Comparative resistance of *Candida albicans* clinical isolates to fluconazole and itraconazole in vitro and in vivo in a murine model. *Antimicrob. Agents Chemother.* **40:**1342–1345.
156. **Vermitsky, J. P., M. J. Self, S. G. Chadwick, J. P. Trama, M. E. Adelson, E. Mordechai, and S. E. Gygax.** 2008. Survey of vaginal-flora *Candida* species isolates from women of different age groups by use of species-specific PCR detection. *J. Clin. Microbiol.* **46:**1501–1503.
157. **Ward, J. I., G. Gorman, C. Phillips, and D. W. Fraser.** 1978. *Hemophilus influenzae* type b disease in a day-care center. Report of an outbreak. *J. Pediatr.* **92:**713–717.
158. **Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel.** 1988. Hospital-acquired candidemia. The attributable mortality and excess length of stay. *Arch. Intern. Med.* **148:**2642–2645.
159. **Wilson, L. S., C. M. Reyes, M. Stolpman, J. Speckman, K. Allen, and J. Beney.** 2002. The direct cost and incidence of systemic fungal infections. *Value Health* **5:**26–34.
160. **Wilton, L., M. Kollarova, E. Heeley, and S. Shakir.** 2003. Relative risk of vaginal candidiasis after use of antibiotics compared with antidepressants in women: postmarketing surveillance data in England. *Drug Saf.* **26:**589–597.
161. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317.
162. **Wisplinghoff, H., H. Seifert, S. M. Tallent, T. Bischoff, R. P. Wenzel, and M. B. Edmond.** 2003. Nosocomial bloodstream infections in pediatric patients in United States hospitals: epidemiology, clinical features and susceptibilities. *Pediatr. Infect. Dis. J.* **22:**686–691.
163. **Wisplinghoff, H., H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2003. Current trends in the epidemiology of nosocomial bloodstream infections in patients with hematological malignancies and solid neoplasms in hospitals in the United States. *Clin. Infect. Dis.* **36:** 1103–1110.
164. **Xu, J., K. Schwartz, M. Bartoces, J. Monsur, R. K. Severson, and J. D. Sobel.** 2008. Effect of antibiotics on vulvovaginal candidiasis: a MetroNet study. *J. Am. Board Fam. Med.* **21:**261–268.
165. **Ye, P., F. H. Rodriguez, S. Kanaly, K. L. Stocking, J. Schurr, P. Schwarzenberger, P. Oliver, W. Huang, P. Zhang, J. Zhang, J. E. Shellito, G. J. Bagby, S. Nelson, K. Charrier, J. J. Peschon, and J. K. Kolls.** 2001. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J. Exp. Med.* **194:**519–527.
166. **Yogev, R., H. B. Lander, and A. T. Davis.** 1978. Effect of TMP-SMX on nasopharyngeal carriage of ampicillin-sensitive and ampicillin-resistant *Hemophilus influenzae* type B. *J. Pediatr.* **93:**394–397.
167. **Zaoutis, T. E., J. Argon, J. Chu, J. A. Berlin, T. J. Walsh, and C. Feudtner.** 2005. The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. *Clin. Infect. Dis.* **41:**1232–1239.
168. **Ziege, S. U., H. F. Geerdes-Fenge, M. Rau, U. Buchwald, and H. Lode.** 2000. In vitro effects of interleukin-10, prednisolone, and GM-CSF on the non-specific immune function of human polymorphonuclear leucocytes and monocytes. *Eur. J. Med. Res.* **5:**369–374.

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch13

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Salivary Histatins: Structure, Function, and Mechanisms of Antifungal Activity

WOONG SIK JANG AND MIRA EDGERTON

HISTATIN OVERVIEW

Saliva is the source of many of the innate nonimmune defense proteins produced in the oral cavity. Salivary proteins play a central role in regulating the oral flora and in preventing overgrowth of *Candida albicans*. However, among over 120 distinct salivary proteins, only a few have antimicrobial activities; and of these, salivary histatins are the primary source of direct fungicidal activity (55, 85). Histatins are a family of histidine-rich proteins secreted by the major salivary glands (submandibular, sublingual, and parotid) and von Ebner's glands in the posterior region of the tongue and in the circumvallate papillae (58). Immunohistochemical studies identified serous cells of the glandular acini as the cells responsible for production of salivary histatins (1). Histatins secreted by the acinar gland complex are released into the salivary ducts, where they undergo further proteolytic modifications before entering the mouth. As a component of whole saliva in the oral cavity, histatins may form heterotypic complexes with other salivary molecules such as mucins, become a part of the salivary coating (or pellicle) of both hard and soft tissues, or be further cleaved into smaller fragments by salivary or bacterial enzymes. Thus, whole saliva contains a series of proteolytic products of histatins in addition to the parent proteins.

Spectrum of Activity

Histatins exhibit fungicidal activity against several *Candida* species (including amphotericin B- and fluconazole-resistant *Candida* spp.) (76), *Aspergillus fumigatus*, some strains of *Saccharomyces cerevisiae*, and *Cryptococcus neoformans* (32). However, histatins are relatively ineffective against most strains of *Candida glabrata* (34), and this resistance is not a result of drug efflux pumps, as *CgCDR1* and *CgCDR2* mutants do not have altered susceptibility to histatin (35). Interestingly, histatin 5 is also effective against the human parasitic protozoan *Leishmania* (49). In contrast to the observed resistance of many biofilm organisms to antimicrobial agents, histatin 5 has antifungal activity against biofilms of *C. albicans* equivalent to that against planktonic cells (42, 59). Thus, histatins may be useful as alternative peptide therapeutics against fluconazole-resistant yeast as well as biofilm forms of fungi.

Cell-Specific Expression of Histatins

Expression of histatins is restricted to human major and minor (von Ebner's) salivary glands (1, 58). Two histatin genes (*HIS2* and *HIS1*) are part of a gene cluster region on human chromosome 4q13 and have evolved from one common ancestral gene (66). *HIS1* (also called *HTN1*) encodes histatin 1, and *HIS2* (also called *HTN3*) encodes histatin 3. This gene family is present only in humans and selected higher primates, although *HIS*-like genes have been identified in cows, and remnants of these genes are present in the mouse and rat at the genomic DNA level (62). Although reverse transcription-PCR analysis detected histatin transcripts in RNA from cow salivary gland tissue, no mouse or rat transcripts have been identified (62). Studies to identify transcriptional regulatory sequences of the *HIS1* gene found that its promoter region contains a positive regulatory element that stimulates transcription strongly in human submandibular salivary gland cells but not in other cell types (36). Also, upstream regulatory elements were identified that may allow cross talk associated with modulated expression of histatins. Thus, transcription of histatins is tissue specific for human salivary gland cells, and its promoter may contain regulatory elements allowing for nonconstitutive expression.

HISTATIN FAMILY MEMBERS

Histatins 1 and 3 (38 and 32 amino acids, respectively) are the full-length parent proteins that undergo maturation and cleavage during and after secretion (Fig. 1). Histatin 1 is phosphorylated on Ser^2, and its tyrosines are variably sulfated by posttranslational modification within the Golgi complex of submandibular and sublingual glands (52). However, histatin 3 contains a substitution of Ala^4 for Glu^4, thus removing its kinase recognition site and preventing phosphorylation of Ser^2 (55, 56). Histatin 1 is cleaved between

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R^{11} and R^{12} to produce histatin 2; however, the parent protein, histatin 1, does not appear to undergo further cleavage (12). In contrast, histatin 3 undergoes multiple sequential cleavages by trypsin-like enzymes to generate a cascade of peptide products. The first proteolytic cleavage is Arg^{25} generating histatin 6 (fragment 1–25), followed by exopeptidase action to produce histatin 5 (fragment 1–24), both occurring during granule maturation. Subsequent proteolytic cleavages of histatin 5 take place after granule secretion and generate a cascade of smaller histatin fragments (7–12). Whole saliva contains a constant ratio of histatin 3 to histatin 5 (31), explained by the requirement for a membrane-bound vesicle protease (31) and an enzyme recognition site in substrate proteins consisting of two Arg residues separated by two amino acids. Interestingly, histatin 1 does not contain these motifs, suggesting a basis for the absence of peptidase cleavage of this protein. Importantly, crucial functional domains within histatins, including antifungal, metal-binding, and wound-healing domains, are largely unaffected by these cleavage events in whole saliva, suggesting a sustained functional activity of these proteins in the proteolytic environment of the oral cavity.

IN VIVO LEVELS OF HISTATINS

The major histatins found in saliva are histatins 1, 3, and 5 (17, 85). Studies of levels of salivary histatins in vivo show large intersubject variation in both the concentrations of histatins and their rates of degradation. However, levels of histatins from the same individual appear to be consistent and are reproducible from day to day. In most instances, large intersubject differences in the concentrations of histatins do not have clinical consequences in people with intact immunity (11). However, human immunodeficiency virus-positive patients were found to have significantly increased levels of total histatins in conjunction with higher levels of oral yeasts (2), although other reports found that histatin 5 concentrations were significantly lower in human immunodeficiency virus-positive patients (75). Similarly, increased fungal carriage has been reported to be associated with either increased levels of salivary histatins (7) or reduced levels of histatins (38). Although these differing results are likely due to the range of disease progression within each patient population, these studies show that there are alterations in histatin levels in saliva in response to disease or perhaps as a result of higher oral fungal burden.

The total concentration of histatins in whole saliva is balanced between secretion of new proteins and removal of "older" proteins by degradation. This is illustrated by the finding that the concentration of histatin collected from parotid glandular secretions is six times higher than in whole saliva due to continual proteolysis within the oral cavity. The sources of proteolytic enzymes in whole saliva are salivary proteases, as well as bacterial (54) and fungal enzymes. At least one *C. albicans*-secreted protease (Sap9) is able to degrade histatin 5 (51), thus suggesting that the presence of *Candida* in the oral cavity may reduce antifungal activity of saliva.

The average total histatin concentration (histatins 1, 3, and 5) in whole saliva is estimated to be 33 ± 16 μg/ml (73). However, the differing susceptibility of each histatin to proteolysis results in uneven distribution levels of the histatins. This was shown by a study in which histatins 1, 3, and 5 added to whole saliva stabilized at concentrations of 5.1, 1.9, and 1.2 μM, respectively, after proteolysis (73). This is in agreement with the reported average in vivo concentrations of histatins in whole saliva from multiple subjects: histatin 1, 19.7 ± 8.4 μg/ml; histatin 3, 5.7 ± 3.5 μg/ml; and histatin 5, 8.0 ± 5.8 μg/ml (28).

The genesis of proteolytic fragments of histatins has functional significance for their fungicidal activity. Histatin 1 is the least effective protein among the family members in its fungicidal and fungistatic activities (85), although it has other roles in binding to tooth surfaces to form a protective pellicle layer (20, 70, 81, 87), preventing adhesion of cariogenic bacteria (69). Histatin 3 has intermediate activity, while histatin 5 has the most potent fungicidal activity among the major histatins (85). Furthermore, proteolytic fragments of histatin 5 including amino acids 11 to 24 (60) and amino acids 4 to 15 (64) were found to have complete activity compared with the parent protein, and even smaller peptides such as fragment 7–15 (27) retained high fungicidal activity (Fig. 1, bottom). Thus, at least half of the known cleavage products of histatin 5 result in formation of bioactive peptides (27), suggesting that the fungicidal activity of saliva is higher than the combined concentrations of histatins 1, 3, and 5.

HISTATIN STRUCTURE

Histatins are rich in basic amino acids, including histidine, arginine, and lysine, resulting in a net cationic charge. To evaluate whether histidine residues are crucial for activity of histatin 5, two adjacent histidine residues (H18 and H19) were replaced with alanines (to avoid altering conformation of the protein), which resulted in reduction of candidacidal activity (76). Although comprehensive substitution analyses of all residues within histatin 5 have not been done, substitution of glutamine for K5 and K13 resulted in nearly complete loss of candidacidal activity, while replacement of adjacent histidine residues had a minimal effect on function (64). Another substitutional study found that K13 was important for candidacidal activity, while R12 and K17 had minimal involvement (77). Interestingly, increasing the amphipathicity of histatin 5 by amino acid substitution had no effect on killing activity, showing that the degree of amphipathic molecular moment of the peptide is unrelated to its fungicidal activity. To further explore the role of amphipathicity for activity, synthetic variants of histatin 5 with increased (dhvar4) and decreased (dhvar5) amphipathic features were examined (33, 65). Both variants were highly active against *C. albicans*, but only the amphipathic variants translocated across and disrupted liposome membranes, while histatin 5 had no interactions with model membranes (16). Thus, unlike many antimicrobial peptides with membranolytic effects, histatin 5 does not interact with model membranes, nor does it rely on amphipathicity for its biological function.

Histatin 5 has no defined secondary structure in aqueous solutions such as saliva, but it does adopt a more helical conformation in dimethyl sulfoxide and aqueous trifluoroethanol (8, 61). To determine whether helical conformation plays a role in the fungicidal activity of histatins, functionally nonessential residues of histatin 5 were replaced with prolines in order to disrupt any helical structure. However, the proline variants had unaltered candidacidal activity (71), showing that helical conformation is not involved in fungicidal activity.

Based upon the structure of histatin 5, several synthetic congeners have been designed and evaluated with regard to their antimicrobial activities against a variety of antibiotic-resistant bacteria and fungi (25, 64). A cysteine-stabilized

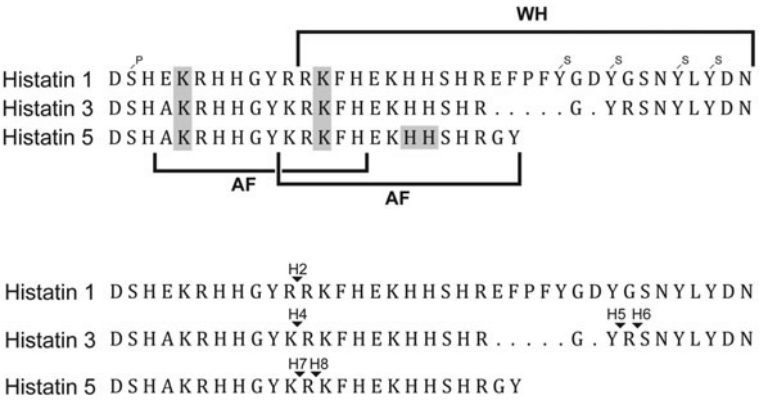

FIGURE 1 Structure and functional domains of histatins. (Top) Primary structure of major histatins 1, 3, and 5. Domains involved in antifungal activity (AF) and wound healing (WH) are bracketed. Shaded amino acids are crucial for antifungal activity. Sulfated (S) and phosphorylated (P) residues are indicated. (Bottom) Cleavage sites that generate other histatin family members (H2, histatin 2; H4, histatin 4; H5, histatin 5; H6, histatin 6; H7, histatin 7; H8, histatin 8) are shown with inverted triangles. 10.1128/9781555817176.ch13f1

cyclized form of histatin 3 was constructed and found to be 100-fold more active against *Saccharomyces cerevisiae* than histatin 5. This activity was attributed to conformation of a looped structure with charged residues protruding on the outside surface, while aromatic residues and histidines were packed into an internal core (9). Like the dhvar4 peptide, this cyclized structural derivative appears to have more disruptive interactions with fungal membranes.

Histatin Interaction with Membranes

Most studies are in agreement that naturally occurring histatins have little to no ability to create transmembrane multimeric peptide pores or disrupt model membranes in vitro (16). Although placement of histatin 5 in a lipid environment was found to promote helical structure formation, it was suggested that ionic and hydrogen bonding interactions between positively charged and polar residues of histatins with the head groups of microbial membranes could be involved (61). However, evidence for in vivo interactions between histatins and fungal cell membranes or organelles is much less clear.

C. *albicans* cells loaded with calcein (a small impermeant dye) did not release this dye upon histatin 5 exposure (21), suggesting that pore formation within the cell membrane does not occur, although cells rapidly lost ATP and small nucleotides as a consequence of histatin toxicity (43). Histatin 5 did not dissipate C. *albicans* cytoplasmic transmembrane potential or uncouple the respiration of isolated mitochondria, also suggesting that its antifungal function does not require membrane pore formation (33). However, other studies have shown that increasing cellular membrane rigidity by exposure of cells to low temperatures or membrane-rigidifying conditions such as energy depletion by azide or the membrane rigidifier Me_2SO is a crucial event in preventing histatin fungicidal activity (80). Veerman et al. further suggest that the physical state of the membrane, possibly mediated by the actin cytoskeleton, influences sensitivity to histatin. Recently, direct examination of cells exposed to histatin 5 by confocal microscopy showed that peptide and propidium iodide initially colocalize to a single site on the cell surface that was interpreted to be the initial point of membrane disruption (53). Monchon and Liu (53) hypothesize that histatin 5 causes a single point of membrane breach that is initiated by binding with an unknown cellular component and that this unitary disruption is the causative event for loss of intracellular ions and cell death. However, other groups have not identified membrane disruption or evidence for single pore formation (15). In reconciling these opposing reports, an important distinction in examining histatins is that very dissimilar effects may be observed depending on peptide dosage as well as the growth phase of the fungal cells.

Histatin Binding to Candidal Cells

Early microscopy studies showed that histatins bind to the cell periphery and then are internalized in a time-dependent manner (86). The toxic effects of histatins occur within the fungal cytosol, as demonstrated by the ability of expressed human histatin 5 from a chromosomally encoded gene in C. *albicans* to induce cell death in the absence of externally applied histatins (3). However, histatins must first associate with and pass through the fungal cell wall in order to reach the cytoplasm.

C. *albicans* cell wall components serve as initial sites of binding with histatin 5 and are important for its uptake, as illustrated by the finding that cell wall digestion to remove surface proteins with β-glucanase resulted in reduced histatin 5 killing (39). In addition, these initial binding interactions with the yeast cell wall are salt sensitive. Histatin 5 (P-113) binding to C. *albicans* was completely inhibited by 100 mM extracellular NaCl (40), thus suggesting that initial binding with the yeast cell wall may involve electrostatic interactions between cationic histatin residues and negatively charged fungal cell wall molecules rather than a receptor-mediated binding. In this regard, histatin killing is pH sensitive (18, 85) and involves achiral interactions, since D-enantiomers of histatins are equally active against C. *albicans* and bacteria (40, 65).

Peptide salt sensitivity as well as initial binding is likely related to charge clustering of basic residues in specific regions of the cationic peptide. Charge interactions between cationic antimicrobial peptides and anionic moieties of cell surfaces can be important determinants for organism specificity as well as salt sensitivity (74). Cationic charge cluster-

ing of dibasic amino acids such as Arg or Lys is commonly found at the N termini of many open-chain antimicrobial peptides. Thus, the number, proximity, and topology of charged amino acids may confer specificity of binding interactions with cell surface molecules. While amphipathicity is not a major factor in salt sensitivity, patterns of hydrogen bonding and hydrophobic interactions between peptides and fungal cell surface glycoproteins may be involved in initial binding.

Binding of histatin 5 with cell surface proteins of *C. albicans* is closely tied to its killing activity and is detected only in histatin 5-sensitive *C. albicans* and *S. cerevisiae* and not in human neutrophil membranes or human embryonic kidney cells (21). This *C. albicans* histatin 5 binding protein was identified as a fungal cell wall-localized heat shock protein, Ssa2 (21, 45). *C. albicans* Ssa2p colocalized with histatin 5 binding protein in vivo, and loss of Ssa2 protein resulted in diminished histatin 5 intracellular transport and cell killing (45). Yeast Ssa proteins are conserved members of the heat shock protein 70 (Hsp70) family that function in heat shock protection, assistance in protein folding, and translocation across membranes independently from the secretory pathway (14). Ssa proteins have been demonstrated to be cell wall-located proteins of both *S. cerevisiae* (48) and *C. albicans* (44, 48) that are capable of inducing cell-mediated immune responses in mice and humans colonized by *C. albicans* (10, 22), thus showing their cell surface position as antigens. Candidal Hsp70 proteins are classical chaperone proteins with two discrete functional domains consisting of peptide binding and ATP binding regions (24). Two contiguous histatin 5 binding sites were identified by both peptide array and limited digestion analyses within ATPase domain, and site-directed mutagenesis of these regions in *C. albicans* Ssa2p confirmed them as histatin 5 binding sites (72). Thus, mutational analyses of Ssa2p showed that this protein contains at least one and perhaps two functional binding sites for histatin 5 that affect intracellular transport and killing by histatin 5.

MECHANISMS OF HISTATIN UPTAKE INTO *C. ALBICANS*

Histatin fungicidal activity is a distinctive multistep mechanism requiring binding to *Candida* cell wall proteins, including Ssap, followed by translocation to intracellular compartments (46). Histatin binding with the cell wall and its intracellular translocation are two independent events, although a minimal level of cell wall binding is a requirement for subsequent uptake. The independence of these two processes is illustrated by the complete blockade of intracellular transport of a substituted histatin 5 fragment, P-113Q2.10 (differing in two amino acids from the active peptide), although it has high levels of cell wall binding (40). Thus, fungal cell wall binding by itself does not result in uptake of histatins.

Intracellular translocation of histatins is inhibited by low temperatures (86), or pretreatment of cells with proton ionophores, including carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and dinitrophenol, or energy metabolism inhibitors such as sodium azide (Fig. 2) (80). Although Veerman et al. noted that azide blocked histatin 5 internalization by cell membrane rigidification due to ATP depletion (80), two other energy-dependent mechanisms for intracellular transport are possible: either active transport of histatin through a cellular transporter or endocytosis (Fig. 3). Endocytosis was initially suggested as a means of histatin cellular entry based upon the observation that bafilomycin, an inhibitor of endosomal acidification, significantly decreased antifungal activity (89). It was suggested that histatins are taken up by endocytosis and subsequently enter the cytosol by their ability to disrupt endosomes and be released through the retrograde pathway. Similarly, cells deficient in Bar domain proteins and defective in endocytosis were also more resistant to the antimicrobial peptide histatin 5, although cells had normal responses to caspofungin and amphotericin (19).

Confocal imaging of *C. albicans* cells showed that some histatin 5 was localized to the vacuole but that cells containing only vacuolar histatin were viable (53). Another study observed histatin 5 trafficking to the vacuole by both vesicle-mediated endocytosis and slower cytoplasm-to-vacuole transport (autophagy) (Fig. 4) (39). However, loss of endocytotic vacuolar trafficking of histatin 5 had no effect on its toxicity, suggesting that vacuolar sequestration or degradation of peptide is not a significant means of removal of toxic peptide from candidal cells (39).

Active transporter-mediated internalization of histatin 5 across the fungal cell membrane is consistent with known requirements for histatin uptake in that input of metabolic energy and a proton motive force are required in a temperature-dependent manner. Since active transport has fairly narrow substrate specificity, polyamine transporters (such as putrescine, spermidine, and spermine) that catalyze the uptake of polycationic molecules may also translocate histatins as polycationic substrates. *C. albicans* Dur transporter proteins are transmembrane proteins with 13 to 15 helices, and kinetic analysis found that Dur3 is a selective transporter for spermidine in *C. albicans* (13). Competitive inhibition for uptake between spermidine and histatin 5 showed that translocation of histatin 5 is through the spermidine-specific transporter Dur3 and that Δ*dur3* cells were significantly resistant to histatin 5 (13). Thus, *C. albicans* Dur3 is a selective plasma membrane transporter for spermidine that also transports histatin 5 into the yeast cytoplasm. Although other transport mechanisms may be utilized for histatin uptake, the Dur family of polyamine transporters provides a significant cytosolic entry mechanism in *C. albicans*.

FUNGICIDAL ACTIVITY OF HISTATIN 5: MITOCHONDRIA

Early work identified *C. albicans* mitochondria as the target of histatin 5 based upon its colocalization with these organelles as well as the resistance of mitochondrial ("petite")

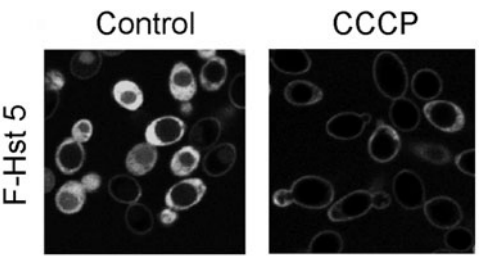

FIGURE 2 Histatin uptake by *C. albicans* is energy dependent. Fluorescein isothiocyanate-labeled histatin 5 (F-Hst 5) added to cells (control) is rapidly translocated to the cytosol, while pretreatment with the protonophore CCCP substantially inhibits cytosolic translocation, although cell wall binding of histatin 5 remains intact. 10.1128/9781555817176.ch13f2

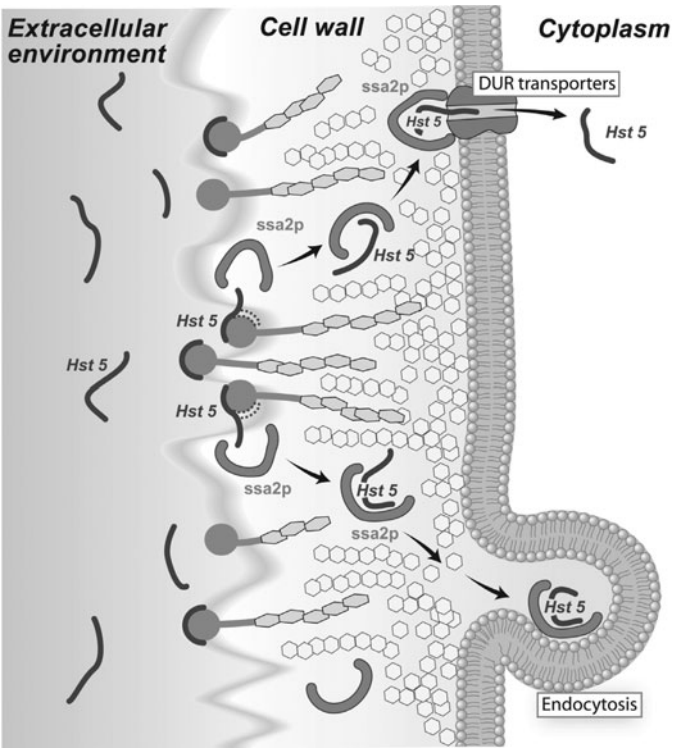

FIGURE 3 Model of histatin binding and uptake by C. *albicans*. Both cell wall polysaccharides and Ssa2 proteins play an important role in initial capture and binding of extracellular histatins. These proteins are facilitators that transfer cell wall-bound histatins (and other cationic peptides) to the actual importer mechanism involving active transport via Dur permeases and energy dependent endocytosis. 10.1128/9781555817176.ch13f3

mutants (26) and cells treated with energy inhibitors such as azide and CCCP to histatin 5 toxicity (26). Thus, an energized cell with unimpaired mitochondrial function supports histatin killing activity. The mechanism of cell death was postulated not to be a result of loss of respiration, since C. *albicans* is fully functional under fermentative (anaerobic) conditions. Instead, the main cause of cell death was generation of toxic levels of reactive oxygen species (ROS) as a consequence of disruption of mitochondrial respiration by histatin (29, 33). However, it has been demonstrated that ROS do not initiate apoptosis, or programmed cell death, pathways in histatin 5-treated cells (84). In addition, others found no role for ROS in histatin 5-induced yeast cell death (79), and subjecting cells to oxidative stress did not enhance histatin 5 killing (82), suggesting that oxidative stress responses are secondary events in toxicity. In this regard, an increase of ROS in *Candida* also has been observed following cell growth arrest (68) and in cells subjected to conditions that cause low intracellular potassium (88). Therefore, ROS may be generated secondarily in C. *albicans* cells as a result of histatin-induced ion loss and cell cycle arrest as well as from mitochondrial damage.

FUNGICIDAL ACTIVITY OF HISTATIN 5: ION AND NUCLEOTIDE EFFLUX

One of the earliest observations about the effects of histatins upon C. *albicans* cells is that upon reaching the cytosol,

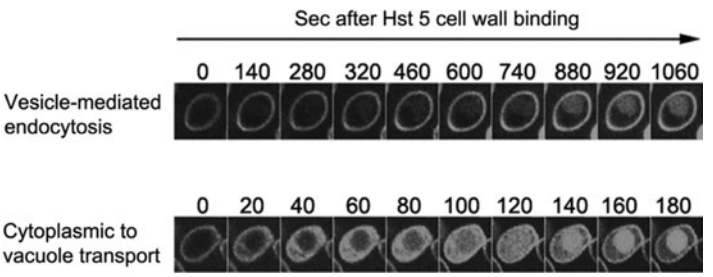

FIGURE 4 Histatin intracellular trafficking utilizes two pathways in C. *albicans*. Two distinct routes of intracellular trafficking of histatin 5 are observed. Endocytotic trafficking directly to the vacuole is slower (top), while cytoplasm-to-vacuole trafficking is more rapid (bottom). Histatin 5 is labeled with fluorescein isothiocyanate for visualization using confocal microscopy (39). 10.1128/9781555817176.ch13f4

histatins induce rapid efflux of cellular ATP and other small nucleotides such as NAD^+, AMP, and ADP, as well as releasing cytosolic potassium and magnesium ions (43, 86). ATP efflux and depletion of intracellular ATP in *C. albicans* cells following histatin 5 treatment has been shown to be concentration and time dependent, with maximal depletion, of about 85%, of intracellular ATP occurring within 30 min of exposure to physiological concentrations of histatin 5 (43). This ion loss then results in an irreversible reduction in total cell volume and then ultimately induces cell cycle arrest (4). The central role of potassium in these events prompted examination of two *C. albicans* potassium channels as potential mediators of histatin 5 toxicity. Knockout of the gene encoding the high-affinity K^+ efflux channel, *TOK1*, had little effect on histatin 5-induced killing of *Candida* (5). However, deletion of *TRK1*, the major plasma membrane K^+ uptake system in *C. albicans*, nearly abolished histatin 5-initiated ATP release, cell killing, and cellular permeability for propidium iodide (PI) (5, 6, 83), indicating that histatin 5 ultimately results in alteration of Trk1p function. Pretreatment of *C. albicans* cells with ion transport inhibitors, such as 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and niflumic acid, blocked ATP loss and killing in histatin 5-treated *C. albicans* (5). Measurement of channel activity showed that DIDS resulted in simultaneous blockade of histatin-induced killing and Trk1p-mediated chloride conductance, providing evidence that Trk1 protein is a critical effector of histatin 5 toxicity in *Candida* (6). Thus, histatin 5 binding to Trk1p produces a leakage pathway through either Trk1 protein itself or a larger complex involving Trk1p. These data suggested a model in which Trk1p is distorted by histatin 5 binding, so that it allows efflux of potassium and larger anions such as ATP. This loss of cytosolic ions subsequently initiates cellular shrinkage and eventual cell cycle arrest, but DIDS "seals" the channel against histatin 5 action. Thus, loss of ability to regulate cellular ionic homeostasis is one crucial feature of histatin fungicidal activity in *C. albicans* cells.

INTRACELLULAR EFFECTS OF HISTATIN 5

The cell wall of *C. albicans* is a thick multilayered structure of glucans, chitin, and mannoproteins that protects cells from osmotic stress and maintains structural integrity (50). Therefore, assessment of histatin-induced cellular effects by ultrastructural analysis reveals little information other than at longer times after more extensive cell damage has occurred. Transmission electron microscopy of cells treated for 1 h with histatins identified vacuole overgrowth, nuclear disappearance, and loss of organelle identity (37). However, more refined methods such as time lapse confocal microscopy showed that histatin 5 rapidly diffused throughout the cytoplasm and was accompanied by expansion of the vacuole in parallel with a loss in cell volume in less than 60 s (53). After 10 min, vacuolar collapse was observed in some cells (53). Jang et al. (39) observed considerable variation in histatin 5 trafficking times among cells, showing that real-time imaging of single cells is valuable for understanding histatin 5 uptake mechanisms. Histatin 5 trafficking to the cytosol and/or vacuole was found to invariably precede PI uptake, marking changes in cell permeability and/or loss of cell integrity. Furthermore, vacuolar accumulation of histatin alone did not result in expansion of this organelle or PI uptake. Instead, PI uptake occurred after cytosolic accumulation of histatin 5 and closely corresponded with vacuolar expansion (39). Since the vacuole is the primary regulator of cell volume and osmolarity by releasing stores of water and ions, the primary function of vacuolar expansion is osmotic recovery. These data suggest a model whereby the primary effect of histatin 5 is induction of osmotic stress through release of intracellular ions and ATP; that is followed by cellular survival responses, including vacuolar expansion and osmotic stress responses (Fig. 5). Furthermore, increasing ionic strength of the extracellular environment prevented killing by intracellular histatin 5 (39), suggesting that histatin 5 activity is mediated by an ion transporter(s) on the yeast plasma membrane.

RESISTANCE TO HISTATINS

Development of resistance to antifungal agents, including azoles and related drugs, is a significant problem for treatment of oral and systemic candidiasis (see chapter 5). A paradigm for use of naturally occurring antifungal peptides (including defensins, histatins, and lactoferrins) as therapeutics is the expectation that development of resistance would be low. However, recent work found that candidal cells may became histatin resistant following successive exposure to histatin (23), showing that cells are capable of dynamic adaptive responses to histatins. Also, histatin resistance phenotypes in *C. albicans* cells have been obtained by cellular energy depletion (26), forcing cells into anaerobic metabolism (29), or in "petite" mutants lacking oxidative respiration (26). However, resistance to histatin and other cationic peptides appears to involve primarily metabolic changes (47), and there is no evidence to support involvement of other mechanisms such as upregulation of drug efflux pumps that carry histatin out of cells.

C. ALBICANS RESPONSE TO HISTATIN 5

To determine how *C. albicans* adapts to the presence of sublethal histatin concentrations, genome-wide gene expression profiling of histatin 5-treated cells was used to gain insight into this peptide's mechanism of action (82). Within 30 min of treatment, 40 genes were significantly altered (24 upregulated and 16 downregulated) in response to histatin 5. Genes significantly increased were the cell stress group (*DDR48*, *HSP90*, and *KAR2*), genes involved in oxidative stress response (*TRX1*, *AHP1*, *SOD5*, and *YHB1*), genes involved in cellular metabolism (mainly the glycolytic pathway), and genes involved in synthesis of cell wall proteins (*PIR1*, *PHR1*, and *KRE6*). Overall transcript profiling of cells surviving histatin 5 treatment showed a marked induction of osmotic and oxidative stress response genes, and many were Hog1 regulated (82). Therefore, activation of one or more of three candidal mitogen-activated protein (MAP) kinase pathways may be involved in stress adaptation, which is important for recovery from histatin 5 toxic effects. Since MAP kinases responsible for protection of *C. albicans* to histatin 5 would result in cell hypersensitivity, *C. albicans* null mutants for MAP kinases Cek1 (ERK-like MAP kinase), Hog1 (high-osmolarity glycerol response), and the Mkc1 kinase (cell wall integrity pathway) were tested for histatin 5 susceptibility. Only *C. albicans hog1*Δ cells were significantly more susceptible to histatin 5, while *C. albicans* Mkc1 and Cek1 knockouts were not significantly different, thus showing that the Hog1 MAP kinase pathway is involved in adaptation and protection of *C. albicans* cells against histatin 5 toxicity (82).

This classical osmotic stress response offers an explanation for why cells grown under anaerobic conditions are more

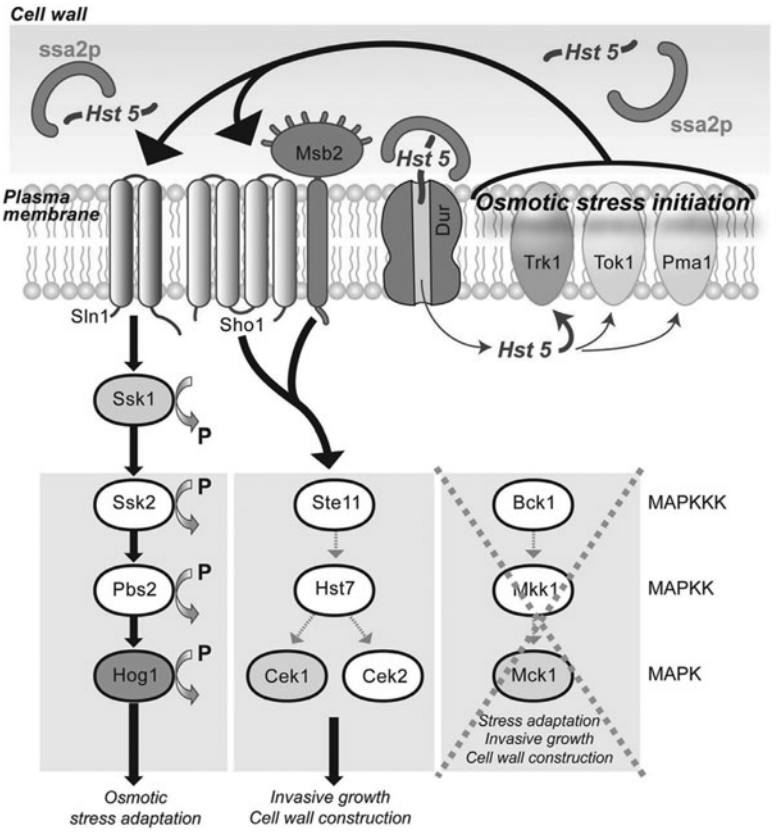

FIGURE 5 Model for histatin toxicity in *C. albicans*. After intracellular entry, histatin causes loss of intracellular ions and nucleotides that are dependent on the presence of Trk1 proteins. Efflux of ions causes osmotic stress-like conditions sensed by the membrane sensors Sho1, Sln1, and Msb2, which initiate signaling by the *C. albicans* Hog1 MAP kinase (or high-osmolarity glycerol) pathway, to allow adaptation to osmotic and oxidative stresses induced by histatins. 10.1128/9781555817176.ch13f5

resistant to histatin 5, since anaerobic growth conditions or selective pressures that force cells into anaerobic growth (such as mitochondrial mutants) are preconditioned for rapid glycerol production and adaptation to osmotic stresses produced by histatin 5. In addition, cells pretreated with osmotic stress are more resistant to subsequent histatin 5 toxicity, similar to conditions used to obtain histatin-resistant mutants. Thus, the high-osmolarity glycerol stress response pathway likely represents a significant and effective challenge to physiological levels of histatins in fungal cells. In the oral cavity, where cationic peptide levels are often transient with respect to colonized candidal cells and histatin 5 concentrations are below 30 μM, the Hog1 response is likely to be a significant and effective challenge to physiological levels of histatin 5. Since *C. albicans* cells do not appear to possess efflux pumps to remove intracellular histatins, adaptive activation of this pathway by cells subjected to prolonged or cyclical histatin stresses is likely a significant mechanism for development of resistance to other toxic cationic peptides.

SUMMARY AND FUTURE DIRECTIONS

A major advantage of the use of histatins and other naturally occurring antifungal proteins for control of oral fungal infections is their innate lack of toxicity. Animal and human clinical studies to evaluate histatins as topical agents in prevention of gingivitis reported therapeutic efficacy without adverse side effects (63, 78). Use of topical histatin preparations or histatin-treated denture acrylic is also being evaluated for controlled release of these antifungal proteins or their active peptide fragments (41, 57). However, a major barrier to the use of cationic peptides as oral therapeutics for oropharyngeal candidiasis is their salt sensitivity. Although saliva is a hypotonic fluid, its ionic strength and unique ionic composition "mask" the candidacidal activity of histatins as a result of inhibited binding between peptide and cell surface (30). Thus, identification of a means to design salt-insensitive fungicidal peptides could greatly enhance their function as "smart" peptides that specifically target yeast cells under physiological conditions. Such peptides would have increased potency against target fungi, thereby reducing dosage and cost and rendering them more attractive for clinical use. Augmentation of innate immunity with naturally occurring peptides such as histatin 5 and human beta defensins is another potential therapy. The major requirements for effective use of such peptides are selective and specific binding and uptake by candidal cells, efficacy at low concentrations that allow rapid eradication of yeast pathogens within the ionic strength of saliva, and minimal fungal resistance. Therefore, the long-range goals of future studies will be to understand mechanisms by which histatin 5 binds and subsequently kills the oral pathogen *C. albicans* in order

to design and optimize peptide-based alternative therapies for oral candidiasis.

REFERENCES

1. **Ahmad, M., M. Piludu, F. G. Oppenheim, E. J. Helmerhorst, and A. R. Hand.** 2004. Immunocytochemical localization of histatins in human salivary glands. *J. Histochem. Cytochem.* **52:**361–370.
2. **Atkinson, J. C., C. Yeh, F. G. Oppenheim, D. Bermudez, B. J. Baum, and P. C. Fox.** 1990. Elevation of salivary antimicrobial proteins following HIV-1 infection. *J. Acquir. Immune Defic. Syndr.* **3:**41–48.
3. **Baev, D., X. Li, and M. Edgerton.** 2001. Genetically engineered human salivary histatin genes are functional in *Candida albicans*: development of a new system for studying histatin candidacidal activity. *Microbiology* **147:**3323–3334.
4. **Baev, D., X. S. Li, J. Dong, P. Keng, and M. Edgerton.** 2002. Human salivary histatin 5 causes disordered volume regulation and cell cycle arrest in *Candida albicans*. *Infect. Immun.* **70:**4777–4784.
5. **Baev, D., A. Rivetta, X. S. Li, S. Vylkova, E. Bashi, C. L. Slayman, and M. Edgerton.** 2003. Killing of *Candida albicans* by human salivary histatin 5 is modulated, but not determined, by the potassium channel TOK1. *Infect. Immun.* **71:**3251–3260.
6. **Baev, D., A. Rivetta, S. Vylkova, J. N. Sun, G. F. Zeng, C. L. Slayman, and M. Edgerton.** 2004. The TRK1 potassium transporter is the critical effector for killing of *Candida albicans* by the cationic protein, Histatin 5. *J. Biol. Chem.* **279:**55060–55072.
7. **Bercier, J. G., I. Al-Hashimi, N. Haghighat, T. D. Rees, and F. G. Oppenheim.** 1999. Salivary histatins in patients with recurrent oral candidiasis. *J. Oral Pathol. Med.* **28:**26–29.
8. **Brewer, D., H. Hunter, and G. Lajoie.** 1998. NMR studies of the antimicrobial salivary peptides histatin 3 and histatin 5 in aqueous and nonaqueous solutions. *Biochem. Cell Biol.* **76:**247–256.
9. **Brewer, D., and G. Lajoie.** 2002. Structure-based design of potent histatin analogues. *Biochemistry* **41:**5526–5536.
10. **Bromuro, C., R. La Valle, S. Sandini, F. Urbani, C. M. Ausiello, L. Morelli, C. Fe d'Ostiani, L. Romani, and A. Cassone.** 1998. A 70-kilodalton recombinant heat shock protein of *Candida albicans* is highly immunogenic and enhances systemic murine candidiasis. *Infect. Immun.* **66:**2154–2162.
11. **Campese, M., X. Sun, J. A. Bosch, F. G. Oppenheim, and E. J. Helmerhorst.** 2009. Concentration and fate of histatins and acidic proline-rich proteins in the oral environment. *Arch. Oral Biol.* **54:**345–353.
12. **Castagnola, M., R. Inzitari, D. V. Rossetti, C. Olmi, T. Cabras, V. Piras, P. Nicolussi, M. T. Sanna, M. Pellegrini, B. Giardina, and I. Messana.** 2004. A cascade of 24 histatins (histatin 3 fragments) in human saliva. Suggestions for a pre-secretory sequential cleavage pathway. *J. Biol. Chem.* **279:**41436–41443.
13. **Chadha, S., J. S. Bajwa, J. N. Sun, J. Paluszynski, W. S. Jang, and M. Edgerton.** 2010. Spermidine plasma membrane transporter Dur31 is involved in the translocation of Histatin 5 in *C. albicans*. *10th ASM Conf. Candida Candidiasis*, abstr. 100B, p. 96.
14. **Craig, E. A., B. D. Gambill, and R. J. Nelson.** 1993. Heat shock proteins: molecular chaperones of protein biogenesis. *Microbiol. Rev.* **57:**402–414.
15. **den Hertog, A. L., J. van Marle, H. A. van Veen, W. Van't Hof, J. G. Bolscher, E. C. Veerman, and A. V. Nieuw Amerongen.** 2005. Candidacidal effects of two antimicrobial peptides: histatin 5 causes small membrane defects, but LL-37 causes massive disruption of the cell membrane. *Biochem. J.* **388:**689–695.
16. **Den Hertog, A. L., H. W. Wong Fong Sang, R. Kraayenhof, J. G. Bolscher, W. Van't Hof, E. C. Veerman, and A. V. Nieuw Amerongen.** 2004. Interactions of histatin 5 and histatin 5-derived peptides with liposome membranes: surface effects, translocation and permeabilization. *Biochem. J.* **379:**665–672.
17. **Denny, P., F. K. Hagen, M. Hardt, L. Liao, W. Yan, M. Arellanno, S. Bassilian, G. S. Bedi, P. Boontheung, D. Cociorva, C. M. Delahunty, T. Denny, J. Dunsmore, K. F. Faull, J. Gilligan, M. Gonzalez-Begne, F. Halgand, S. C. Hall, X. Han, B. Henson, J. Hewel, S. Hu, S. Jeffrey, J. Jiang, J. A. Loo, R. R. Ogorzalek Loo, D. Malamud, J. E. Melvin, O. Miroshnychenko, M. Navazesh, R. Niles, S. K. Park, A. Prakobphol, P. Ramachandran, M. Richert, S. Robinson, M. Sondej, P. Souda, M. A. Sullivan, J. Takashima, S. Than, J. Wang, J. P. Whitelegge, H. E. Witkowska, L. Wolinsky, Y. Xie, T. Xu, W. Yu, J. Ytterberg, D. T. Wong, J. R. Yates III, and S. J. Fisher.** 2008. The proteomes of human parotid and submandibular/sublingual gland salivas collected as the ductal secretions. *J. Proteome Res.* **7:**1994–2006.
18. **Dong, J., S. Vylkova, X. S. Li, and M. Edgerton.** 2003. Calcium blocks fungicidal activity of human salivary histatin 5 through disruption of binding with *Candida albicans*. *J. Dent. Res.* **82:**748–752.
19. **Douglas, L. M., S. W. Martin, and J. B. Konopka.** 2009. BAR domain proteins Rvs161 and Rvs167 contribute to *Candida albicans* endocytosis, morphogenesis, and virulence. *Infect. Immun.* **77:**4150–4160.
20. **Driscoll, J., Y. Zuo, T. Xu, J. R. Choi, R. F. Troxler, and F. G. Oppenheim.** 1995. Functional comparison of native and recombinant human salivary histatin 1. *J. Dent. Res.* **74:**1837–1844.
21. **Edgerton, M., S. E. Koshlukova, T. E. Lo, B. G. Chrzan, R. M. Straubinger, and P. A. Raj.** 1998. Candidacidal activity of salivary histatins. Identification of a histatin 5-binding protein on *Candida albicans*. *J. Biol. Chem.* **273:**20438–20447.
22. **Eroles, P., M. Sentandreu, M. V. Elorza, and R. Sentandreu.** 1997. The highly immunogenic enolase and Hsp70p are adventitious *Candida albicans* cell wall proteins. *Microbiology* **143**(Pt. 2)**:**313–320.
23. **Fitzgerald-Hughes, D. H., D. C. Coleman, and B. C. O'Connell.** 2007. Differentially expressed proteins in derivatives of *Candida albicans* displaying a stable histatin 3-resistant phenotype. *Antimicrob. Agents Chemother.* **51:** 2793–2800.
24. **Flaherty, K. M., C. DeLuca-Flaherty, and D. B. McKay.** 1990. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature* **346:**623–628.
25. **Giacometti, A., O. Cirioni, W. Kamysz, G. D'Amato, C. Silvestri, M. S. Del Prete, A. Licci, A. Riva, J. Lukasiak, and G. Scalise.** 2005. In vitro activity of the histatin derivative P-113 against multidrug-resistant pathogens responsible for pneumonia in immunocompromised patients. *Antimicrob. Agents Chemother.* **49:**1249–1252.
26. **Gyurko, C., U. Lendenmann, R. F. Troxler, and F. G. Oppenheim.** 2000. *Candida albicans* mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5. *Antimicrob. Agents Chemother.* **44:**348–354.
27. **Hardt, M., L. R. Thomas, S. E. Dixon, G. Newport, N. Agabian, A. Prakobphol, S. C. Hall, H. E. Witkowska, and S. J. Fisher.** 2005. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. *Biochemistry* **44:**2885–2899.

28. **Helmerhorst, E. J., A. S. Alagl, W. L. Siqueira, and F. G. Oppenheim.** 2006. Oral fluid proteolytic effects on histatin 5 structure and function. *Arch. Oral Biol.* **51:**1061–1070.
29. **Helmerhorst, E. J., P. Breeuwer, W. van't Hof, E. Walgreen-Weterings, L. C. Oomen, E. C. Veerman, A. V. Amerongen, and T. Abee.** 1999. The cellular target of histatin 5 on *Candida albicans* is the energized mitochondrion. *J. Biol. Chem.* **274:**7286–7291.
30. **Helmerhorst, E. J., B. Flora, R. F. Troxler, and F. G. Oppenheim.** 2004. Dialysis unmasks the fungicidal properties of glandular salivary secretions. *Infect. Immun.* **72:**2703–2709.
31. **Helmerhorst, E. J., and F. G. Oppenheim.** 2007. Saliva: a dynamic proteome. *J. Dent. Res.* **86:**680–693.
32. **Helmerhorst, E. J., I. M. Reijnders, W. van't Hof, I. Simoons-Smit, E. C. Veerman, and A. V. Amerongen.** 1999. Amphotericin B- and fluconazole-resistant *Candida* spp., *Aspergillus fumigatus*, and other newly emerging pathogenic fungi are susceptible to basic antifungal peptides. *Antimicrob. Agents Chemother.* **43:**702–704.
33. **Helmerhorst, E. J., R. F. Troxler, and F. G. Oppenheim.** 2001. The human salivary peptide histatin 5 exerts its antifungal activity through the formation of reactive oxygen species. *Proc. Natl. Acad. Sci. USA* **98:**14637–14642.
34. **Helmerhorst, E. J., C. Venuleo, A. Beri, and F. G. Oppenheim.** 2005. *Candida glabrata* is unusual with respect to its resistance to cationic antifungal proteins. *Yeast* **22:** 705–714.
35. **Helmerhorst, E. J., C. Venuleo, D. Sanglard, and F. G. Oppenheim.** 2006. Roles of cellular respiration, CgCDR1, and CgCDR2 in *Candida glabrata* resistance to histatin 5. *Antimicrob. Agents Chemother.* **50:**1100–1103.
36. **Imamura, Y., Y. Fujigaki, Y. Oomori, K. Ouryouji, S. Yanagisawa, H. Miyazawa, and P. L. Wang.** 2009. Transcriptional regulation of the salivary histatin gene: finding of a strong positive regulatory element and its binding protein. *J. Biochem.* **145:**279–288.
37. **Isola, R., M. Isola, G. Conti, M. S. Lantini, and A. Riva.** 2007. Histatin-induced alterations in *Candida albicans*: a microscopic and submicroscopic comparison. *Microsc. Res. Tech.* **70:**607–616.
38. **Jainkittivong, A., D. A. Johnson, and C. K. Yeh.** 1998. The relationship between salivary histatin levels and oral yeast carriage. *Oral Microbiol. Immunol.* **13:**181–187.
39. **Jang, W. S., J. S. Bajwa, J. N. Sun, and M. Edgerton.** 2010. Salivary histatin 5 internalization by translocation, but not endocytosis, is required for fungicidal activity in *Candida albicans*. *Mol. Microbiol.* **77:**354–370.
40. **Jang, W. S., X. S. Li, J. N. Sun, and M. Edgerton.** 2008. The P-113 fragment of histatin 5 requires a specific peptide sequence for intracellular translocation in *Candida albicans*, which is independent of cell wall binding. *Antimicrob. Agents Chemother.* **52:**497–504.
41. **Kavanagh, K., and S. Dowd.** 2004. Histatins: antimicrobial peptides with therapeutic potential. *J. Pharm. Pharmacol.* **56:**285–289.
42. **Konopka, K., B. Dorocka-Bobkowska, S. Gebremedhin, and N. Duzgunes.** 2010. Susceptibility of *Candida* biofilms to histatin 5 and fluconazole. *Antonie van Leeuwenhoek* **97:**413–417.
43. **Koshlukova, S. E., T. L. Lloyd, M. W. Araujo, and M. Edgerton.** 1999. Salivary histatin 5 induces non-lytic release of ATP from *Candida albicans* leading to cell death. *J. Biol. Chem.* **274:**18872–18879.
44. **La Valle, R., C. Bromuro, L. Ranucci, H. M. Muller, A. Crisanti, and A. Cassone.** 1995. Molecular cloning and expression of a 70-kilodalton heat shock protein of *Candida albicans*. *Infect. Immun.* **63:**4039–4045.
45. **Li, X. S., M. S. Reddy, D. Baev, and M. Edgerton.** 2003. *Candida albicans* Ssa1/2p is the cell envelope binding protein for human salivary histatin 5. *J. Biol. Chem.* **278:** 28553–28561.
46. **Li, X. S., J. N. Sun, K. Okamoto-Shibayama, and M. Edgerton.** 2006. *Candida albicans* cell wall Ssa proteins bind and facilitate import of salivary histatin 5 required for toxicity. *J. Biol. Chem.* **281:**22453–22463.
47. **Lis, M., and L. A. Bobek.** 2008. Proteomic and metabolic characterization of a *Candida albicans* mutant resistant to the antimicrobial peptide MUC7 12-mer. *FEMS Immunol. Med. Microbiol.* **54:**80–91.
48. **Lopez-Ribot, J. L., H. M. Alloush, B. J. Masten, and W. L. Chaffin.** 1996. Evidence for presence in the cell wall of *Candida albicans* of a protein related to the hsp70 family. *Infect. Immun.* **64:**3333–3340.
49. **Luque-Ortega, J. R., W. van't Hof, E. C. Veerman, J. M. Saugar, and L. Rivas.** 2008. Human antimicrobial peptide histatin 5 is a cell-penetrating peptide targeting mitochondrial ATP synthesis in *Leishmania*. *FASEB J.* **22:**1817–1828.
50. **Martinez, J. P., M. L. Gil, J. L. Lopez-Ribot, and W. L. Chaffin.** 1998. Serologic response to cell wall mannoproteins and proteins of *Candida albicans*. *Clin. Microbiol. Rev.* **11:**121–141.
51. **Meiller, T. F., B. Hube, L. Schild, M. E. Shirtliff, M. A. Scheper, R. Winkler, A. Ton, and M. A. Jabra-Rizk.** 2009. A novel immune evasion strategy of *Candida albicans*: proteolytic cleavage of a salivary antimicrobial peptide. *PLoS One* **4:**e5039.
52. **Messana, I., T. Cabras, E. Pisano, M. T. Sanna, A. Olianas, B. Manconi, M. Pellegrini, G. Paludetti, E. Scarano, A. Fiorita, S. Agostino, A. M. Contucci, L. Calo, P. M. Picciotti, A. Manni, A. Bennick, A. Vitali, C. Fanali, R. Inzitari, and M. Castagnola.** 2008. Trafficking and postsecretory events responsible for the formation of secreted human salivary peptides: a proteomics approach. *Mol. Cell. Proteomics* **7:**911–926.
53. **Mochon, A. B., and H. Liu.** 2008. The antimicrobial peptide histatin-5 causes a spatially restricted disruption on the *Candida albicans* surface, allowing rapid entry of the peptide into the cytoplasm. *PLoS Pathog.* **4:**e1000190.
54. **O'Brien-Simpson, N. M., S. G. Dashper, and E. C. Reynolds.** 1998. Histatin 5 is a substrate and not an inhibitor of the Arg- and Lys-specific proteinases of *Porphyromonas gingivalis*. *Biochem. Biophys. Res. Commun.* **250:**474–478.
55. **Oppenheim, F. G., T. Xu, F. M. McMillian, S. M. Levitz, R. D. Diamond, G. D. Offner, and R. F. Troxler.** 1988. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on *Candida albicans*. *J. Biol. Chem.* **263:**7472–7477.
56. **Oppenheim, F. G., Y. C. Yang, R. D. Diamond, D. Hyslop, G. D. Offner, and R. F. Troxler.** 1986. The primary structure and functional characterization of the neutral histidine-rich polypeptide from human parotid secretion. *J. Biol. Chem.* **261:**1177–1182.
57. **Paquette, D. W., D. M. Simpson, P. Friden, V. Braman, and R. C. Williams.** 2002. Safety and clinical effects of topical histatin gels in humans with experimental gingivitis. *J. Clin. Periodontol.* **29:**1051–1058.
58. **Piludu, M., M. S. Lantini, M. Cossu, M. Piras, F. G. Oppenheim, E. J. Helmerhorst, W. Siqueira, and A. R. Hand.** 2006. Salivary histatins in human deep posterior lingual glands (of von Ebner). *Arch. Oral Biol.* **51:**967–973.
59. **Pusateri, C. R., E. A. Monaco, and M. Edgerton.** 2009. Sensitivity of *Candida albicans* biofilm cells grown on denture acrylic to antifungal proteins and chlorhexidine. *Arch. Oral Biol.* **54:**588–594.
60. **Raj, P. A., M. Edgerton, and M. J. Levine.** 1990. Salivary histatin 5: dependence of sequence, chain length, and

helical conformation for candidacidal activity. *J. Biol. Chem.* **265:**3898–3905.
61. **Raj, P. A., E. Marcus, and D. K. Sukumaran.** 1998. Structure of human salivary histatin 5 in aqueous and non-aqueous solutions. *Biopolymers* **45:**51–67.
62. **Rijnkels, M., L. Elnitski, W. Miller, and J. M. Rosen.** 2003. Multispecies comparative analysis of a mammalian-specific genomic domain encoding secretory proteins. *Genomics* **82:**417–432.
63. **Rothstein, D. M., E. J. Helmerhorst, P. Spacciapoli, F. G. Oppenheim, and P. Friden.** 2002. Histatin-derived peptides: potential agents to treat localised infections. *Expert Opin. Emerg. Drugs* **7:**47–59.
64. **Rothstein, D. M., P. Spacciapoli, L. T. Tran, T. Xu, F. D. Roberts, M. Dalla Serra, D. K Buxton, F. G. Oppenheim, and P. Friden.** 2001. Anticandidal activity is retained in P-113, a 12-amino-acid fragment of histatin 5. *Antimicrob. Agents Chemother.* **45:**1367–1373.
65. **Ruissen, A. L., J. Groenink, E. J. Helmerhorst, E. Walgreen-Weterings, W. Van't Hof, E. C. Veerman, and A. V. Nieuw Amerongen.** 2001. Effects of histatin 5 and derived peptides on *Candida albicans*. *Biochem. J.* **356:**361–368.
66. **Sabatini, L. M., and E. A. Azen.** 1989. Histatins, a family of salivary histidine-rich proteins, are encoded by at least two loci (HIS1 and HIS2). *Biochem. Biophys. Res. Commun.* **160:**495–502.
67. **Sajjan, U. S., L. T. Tran, N. Sole, C. Rovaldi, A. Akiyama, P. M. Friden, J. F. Forstner, and D. M. Rothstein.** 2001. P-113D, an antimicrobial peptide active against *Pseudomonas aeruginosa*, retains activity in the presence of sputum from cystic fibrosis patients. *Antimicrob. Agents Chemother.* **45:**3437–3444.
68. **Sander, C. S., U. C. Hipler, U. Wollina, and P. Elsner.** 2002. Inhibitory effect of terbinafine on reactive oxygen species (ROS) generation by *Candida albicans*. *Mycoses* **45:**152–155.
69. **Shimotoyodome, A., H. Kobayashi, I. Tokimitsu, T. Matsukubo, and Y. Takaesu.** 2006. Statherin and histatin 1 reduce parotid saliva-promoted *Streptococcus mutans* strain MT8148 adhesion to hydroxyapatite surfaces. *Caries Res.* **40:**403–411.
70. **Siqueira, W. L., H. C. Margolis, E. J. Helmerhorst, F. M. Mendes, and F. G. Oppenheim.** 2010. Evidence of intact histatins in the in vivo acquired enamel pellicle. *J. Dent. Res.* **29:**29.
71. **Situ, H., S. V. Balasubramanian, and L. A. Bobek.** 2000. Role of alpha-helical conformation of histatin-5 in candidacidal activity examined by proline variants. *Biochim. Biophys. Acta* **1475:**377–382.
72. **Sun, J. N., W. Li, W. S. Jang, N. Nayyar, M. D. Sutton, and M. Edgerton.** 2008. Uptake of the antifungal cationic peptide Histatin 5 by *Candida albicans* Ssa2p requires binding to non-conventional sites within the ATPase domain. *Mol. Microbiol.* **70:**1246–1260.
73. **Sun, X., E. Salih, F. G. Oppenheim, and E. J. Helmerhorst.** 2009. Kinetics of histatin proteolysis in whole saliva and the effect on bioactive domains with metal-binding, antifungal, and wound-healing properties. *FASEB J.* **23:**2691–2701.
74. **Tam, J. P., Y. A. Lu, and J. L. Yang.** 2002. Correlations of cationic charges with salt sensitivity and microbial specificity of cystine-stabilized beta-strand antimicrobial peptides. *J. Biol. Chem.* **277:**50450–50456.
75. **Torres, S. R., A. Garzino-Demo, T. F. Meiller, V. Meeks, and M. A. Jabra-Rizk.** 2009. Salivary histatin-5 and oral fungal colonisation in HIV+ individuals. *Mycoses* **52:**11–15.
76. **Tsai, H., and L. A. Bobek.** 1997. Studies of the mechanism of human salivary histatin-5 candidacidal activity with histatin-5 variants and azole-sensitive and -resistant *Candida* species. *Antimicrob. Agents Chemother.* **41:**2224–2228.
77. **Tsai, H., P. A. Raj, and L. A. Bobek.** 1996. Candidacidal activity of recombinant human salivary histatin-5 and variants. *Infect. Immun.* **64:**5000–5007.
78. **Van Dyke, T., D. Paquette, S. Grossi, V. Braman, J. Massaro, R. D'Agostino, S. Dibart, and P. Friden.** 2002. Clinical and microbial evaluation of a histatin-containing mouthrinse in humans with experimental gingivitis: a phase-2 multi-center study. *J. Clin. Periodontol.* **29:**168–176.
79. **Veerman, E. C., K. Nazmi, W. Van't Hof, J. G. Bolscher, A. L. Den Hertog, and A. V. Nieuw Amerongen.** 2004. Reactive oxygen species play no role in the candidacidal activity of the salivary antimicrobial peptide histatin 5. *Biochem. J.* **381:**447–452.
80. **Veerman, E. C., M. Valentijn-Benz, K. Nazmi, A. L. Ruissen, E. Walgreen-Weterings, J. van Marle, A. B. Doust, W. van't Hof, J. G. Bolscher, and A. V. Amerongen.** 2007. Energy depletion protects *Candida albicans* against antimicrobial peptides by rigidifying its cell membrane. *J. Biol. Chem.* **282:**18831–18841.
81. **Vitorino, R., M. J. Calheiros-Lobo, J. A. Duarte, P. M. Domingues, and F. M. Amado.** 2008. Peptide profile of human acquired enamel pellicle using MALDI tandem MS. *J. Sep. Sci.* **31:**523–537.
82. **Vylkova, S., W. S. Jang, W. Li, N. Nayyar, and M. Edgerton.** 2007. Histatin 5 initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway. *Eukaryot. Cell* **6:** 1876–1888.
83. **Vylkova, S., X. S. Li, J. C. Berner, and M. Edgerton.** 2006. Distinct antifungal mechanisms: beta-defensins require *Candida albicans* Ssa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. *Antimicrob. Agents Chemother.* **50:**324–331.
84. **Wunder, D., J. Dong, D. Baev, and M. Edgerton.** 2004. Human salivary histatin 5 fungicidal action does not induce programmed cell death pathways in *Candida albicans*. *Antimicrob. Agents Chemother.* **48:**110–115.
85. **Xu, T., S. M. Levitz, R. D. Diamond, and F. G. Oppenheim.** 1991. Anticandidal activity of major human salivary histatins. *Infect. Immun.* **59:**2549–2554.
86. **Xu, Y., I. Ambudkar, H. Yamagishi, W. Swaim, T. J. Walsh, and B. C. O'Connell.** 1999. Histatin 3-mediated killing of *Candida albicans*: effect of extracellular salt concentration on binding and internalization. *Antimicrob. Agents Chemother.* **43:**2256–2262.
87. **Yin, A., H. C. Margolis, J. Grogan, Y. Yao, R. F. Troxler, and F. G. Oppenheim.** 2003. Physical parameters of hydroxyapatite adsorption and effect on candidacidal activity of histatins. *Arch. Oral Biol.* **48:**361–368.
88. **Zhou, X., W. Yin, S. Q. Doi, S. W. Robinson, K. Takeyasu, and X. Fan.** 2003. Stimulation of Na,K-ATPase by low potassium requires reactive oxygen species. *Am. J. Physiol. Cell Physiol.* **285:**C319–C326.
89. **Zhu, J., P. W. Luther, Q. Leng, and A. J. Mixson.** 2006. Synthetic histidine-rich peptides inhibit *Candida* species and other fungi in vitro: role of endocytosis and treatment implications. *Antimicrob. Agents Chemother.* **50:** 2797–2805.

HOST-PATHOGEN INTERACTIONS (THE PATHOGEN)

III

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch14

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The Cell Wall: Glycoproteins, Remodeling, and Regulation

CAROL MUNRO AND MATHIAS L. RICHARD

INTRODUCTION

The fungal cell wall is a sticky, protective, carbohydrate-rich sheath exterior to the cell membrane. The cell wall is a dynamic organelle that can be restructured and reinforced by a number of coordinated mechanisms in response to a variety of signals. It forms the interface with the environment, and in pathogenic fungi this includes interactions with the human host as well as the host's resident microbiota. As a consequence, cell wall constituents, both saccharide and protein, play roles in a number of stages of the infection process from adhesion and colonization, formation of biofilms, invasion of host tissues, resistance to the host's oxidative burst, antifungal drug susceptibility, and interaction with and modulation of the host's immune response. A robust cell wall is vital to maintain viability by resisting internal turgor pressure, yet the wall is malleable and can be restructured to alter cell shape during morphogenesis. Alterations in cell wall architecture and composition are also brought about by a variety of extracellular and intracellular signals, including response to cell wall-damaging agents and genetic mutations. The main objective of this chapter is to provide an update on cell wall structure and, in particular, cell wall glycoproteins (CWPs), cell wall remodeling, and regulation. Readers should also see chapter 16 for additional insights.

CELL WALL POLYSACCHARIDES

Electron micrographs of the *Candida* cell wall reveal an electron-translucent layer proximal to the plasma membrane and an electron-dense layer on the exterior that is elaborated with thread-like filaments or fibrils that extend outwards (Fig. 1). The electron-translucent layer is composed of chitin and β(1,3)-glucan, the main structural polysaccharides of the cell wall. The outer layer is composed of highly glycosylated proteins that are attached via β(1,6)-linked glucan to β(1,3)-glucan (86, 87). β(1,3)-Glucan is the main constituent of wild-type *Candida* cells, comprising 40% of the wall; β(1,6)-glucan makes up 20% of the wall, chitin makes up to 5%, and the remainder is glycoproteins, composed mainly of mannose-rich glycan chains attached to the proteins (20).

Chitin Synthesis

Chitin is a linear homopolymer of β(1,4)-linked *N*-acetylglucosamine residues. Nascent chains of chitin become cross-linked to each other in an antiparallel fashion to form crystalline fibrils. In fungi, chitin is synthesised by a family of membrane-localized chitin synthase enzymes best characterized in *Saccharomyces cerevisiae*, with three members (Chs1, Chs2, and Chs3), and *Candida albicans*, which has four members (Chs1, Chs2, Chs3, and Chs8) (145). Chitin synthase binds UDP-*N*-acetylglucosamine on the cytoplasmic face of the plasma membrane, *N*-acetylglucosamine residues are joined by β(1,4) linkages, and the chitin chain is extruded out from the plasma membrane towards the cell wall. Chitin is covalently attached to the nonreducing ends of β(1,3)-glucan (98, 99). The Chs enzymes are under spatial and temporal regulation and can form chitin microfibrils with different architectures: Chs8 makes long fibrils and Chs3 produces the majority of chitin found in the wall, which resembles short rodlets (109). *CHS1*, *CHS3*, and *CHS8* are under cell cycle regulation (36), and Chs2, Chs3, and Chs8 are phosphorylated (107). Mutation of the phosphorylation site of Chs3 interferes with its normal localization pattern (107). The protein kinase C (PKC), high-osmolarity glycerol (HOG), and Ca^{2+}/calcineurin signaling pathways have all been implicated in the regulation of chitin synthesis in *C. albicans* (147). The role of fungal cell wall chitin in the immune response to fungal pathogens remains poorly understood (see chapter 11), but chitin has been implicated as an immunomodulatory molecule in other systems (reviewed in reference 108).

β(1,3)-Glucan

β(1,3)-Glucan is a polymer of glucose residues that is synthesised from UDP-glucose by the integral membrane

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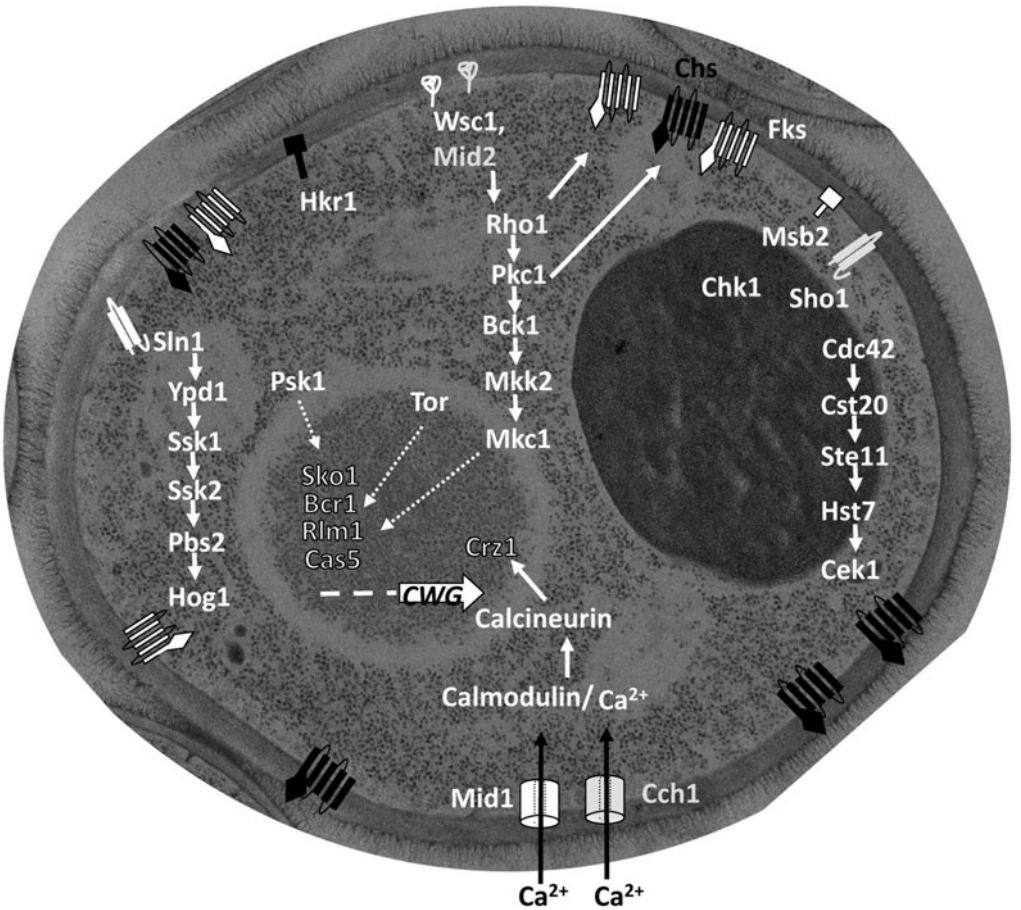


FIGURE 1 Signaling pathways that regulate cell wall biogenesis and remodeling. The cross-section of a *C. albicans* yeast cell prepared by high-pressure freeze substitution transmission electron microscopy demonstrates the outer fibrillar cell surface composed of highly glycosylated glycoproteins surrounding an electron transparent polysaccharide-rich matrix made mainly of glucan with some chitin. In *S. cerevisiae*, several integral membrane proteins with glycosylated extracellular domains (Wsc family, Mid2, Mtl1, Msb2, Hkr1, Sho1, and Sln1) act as sensors that respond to changes in cell wall integrity and activate downstream signaling pathways. Msb2, Hkr1, Sln1, and Sho1 also act as osmosensors. In *C. albicans*, Msb2 and Sho1 signal to the CEK pathway and Sln1 signals to the HOG pathway. In *C. albicans*, the Chk1 histidine kinase and the CEK pathway regulate cell wall biosynthesis, and *chk1*Δ and *cek1*Δ mutants have defects in mannan biosynthesis. The PKC pathway is the classical pathway that maintains cell integrity by regulating cell wall biogenesis and remodeling. In *S. cerevisiae*, the Wsc and Mid2 sensors signal to Rho1, which activates Pkc1 and the downstream MAP kinase cascade culminating in ScSlt2 phosphorylation, and in *C. albicans*, the orthologous MAP kinase is Mkc1. In addition, in *S. cerevisiae*, Rho1 acts as a regulatory subunit of β(1,3)-glucan synthase and Pkc1 is involved in targeting Chs3 to the plasma membrane in response to heat shock. A number of transcription factors, outlined in black, have been identified in *C. albicans* as regulating the expression of cell wall-related genes (*CWG*). Cas5 and Sko1 are involved in the response to echinocandins, and Bcr1 regulates the expression of a number of important adhesins and functions downstream of the TOR signaling pathway. The role of *Ca*Sko1 in the transcriptional response to caspofungin is dependent on the Psk1 PAS kinase. Crz1 is the transcription factor that lies downstream of the Ca^{2+}/calmodulin pathway. In response to elevated intracellular Ca^{2+} levels, Crz1 becomes dephosphorylated by calcineurin, moves into the nucleus, and activates expression of genes with calcium-dependent response elements within their promoter sequences. In *C. albicans* the genes encoding the Crh GPI-anchored protein family are examples of calcium-responsive genes. The PKC, HOG, and Ca^{2+} signaling all contribute to the regulation of chitin synthesis in *C. albicans*. The signaling pathways are based on the *S. cerevisiae* paradigm and are predicted to have homologous functions in *C. albicans*. In some cases rewiring of pathway components has been experimentally demonstrated in *C. albicans* and is presented in the schematic. 10.1128/9781555817176.ch14f1

protein β(1,3)-glucan synthase. The glucan synthase complex is composed of a catalytic subunit (Fks/Gsc) and a regulatory subunit (Rho1) (100, 134, 171). Rho1 is a small Rho-type GTPase that is activated when GTP is bound and inactive when bound to GDP. In *S. cerevisiae*, Hkr1p and Smi1p/Knr4p are also involved in β(1,3)-glucan synthesis; Hkr1 is a mucin thought to act as an osmosensor in the Sho1 branch of the HOG pathway, while Smi1/Knr4 physically interacts with Slt2 and is required for PKC pathway signaling (74, 132, 167, 217). Fungi tend to have more than one β(1,3)-glucan synthase catalytic subunit; for example, in *S. cerevisiae*, Fks1 is responsible for glucan synthesis during vegetative growth, and Fks2 and Fks3 are active during sporulation (83). In *C. albicans*, Fks1/Gsc1 is the main β(1,3)-glucan synthase activity and the target for echinocandin drugs (44). Point mutations within the *FKS* genes have been identified that result in echinocandin resistance in a variety of *Candida* species (162, 210). In addition, β(1,3)-glucan is an important ligand or pathogen-associated molecular pattern recognized and bound by the pattern recognition receptor dectin-1, found on cells of the innate immune response (discussed in chapter 11) (19, 176).

β(1,6)-Glucan

The synthesis of β(1,6)-glucan remains poorly understood. In *S. cerevisiae*, a number of genes—*BIG1*, *KRE1*, *KRE6*, *KRE9*, *KRE11*, and *ROT1*—have been shown to contribute to β(1,6)-glucan levels in the cell wall (190). Similarly, the *C. albicans big1* mutant had decreased β(1,6)-glucan levels (205). *S. cerevisiae* β(1,6)-glucan was recently characterized in more detail and found to be 38 kDa in size and with β(1,3)-linked side chains of one or two glucose residues approximately every fifth residue (1). However, *S. cerevisiae* has much lower levels of β(1,6)-glucan in its wall, around 5 to 10%, than *C. albicans*, where β(1,6)-glucan makes up around one-fifth of the cell wall (197). The two species have similar repertoires of genes required for β(1,6)-glucan synthesis, although *C. albicans* lacks a clear Kre11 orthologue. Using a different approach, Iorio et al. showed that *C. albicans* also has a β(1,6)-linked glucan polymer with β(1,3)-linked glucose side branches (82).

Cell Wall-Localized Carbohydrate-Active Enzymes

Although we understand how the main structural polysaccharides of the wall are synthesized, we have little understanding of how the different components are assembled into the mature wall. However, we do know that a number of wall-associated proteins have activities that can modify and process cell wall carbohydrates (see below and Table 1). These carbohydrate-active enzymes (26) (see http://www.cazy.org/) are conserved in fungal species, including the CTG clade species, *S. cerevisiae*, and *Candida glabrata* (21, 39). Included are the Gas family proteins, which modify β(1,3)-glucan chains; the Crh family proteins, which cross-link chitin to glucan (24, 25); and a number of glucanases (Table 1).

The first fungal β(1,6)–β(1,3)-glucan branching transglycosidase activity was recently identified in *Aspergillus fumigatus* and is encoded by *AfBGT2*. This novel enzyme was capable of removing laminaribose from the reducing end of a β(1,3)-linked glucose oligosaccharide chain and attaching it to an internal acceptor at carbon 6, forming a β(1,3)-glucan chain with a β(1,6) side chain. However, the *afbgt2* mutant did not display any cell wall defects (67).

TABLE 1 *C. albicans* cell wall-associated carbohydrate-active enzymes

Common name	Gene identity	Activity	Family	CAZy family[a]
Bgl2	orf19.4565	Exo-β(1,3)-glucanase	Scw-like	GH17
	orf19.4668			
MP65/Scw1	orf19.1779			
Scw4	orf19.2941			
Phr1	orf19.3829	β(1,3)-Glucan elongases/transglycosidases	Gas	GH72
Phr2	orf19.6081			
Phr3	orf19.377			
Pga4	orf19.4035			
Pga5	orf19.3693			
Crh11	orf19.2706	Chitin β(1,6)-glucanosyltransferase	Crh	GH16
Crh12	orf19.3966			
Utr2	orf19.1671			
Cht1	orf19.7517	Chitinases	Cht	GH18
Cht2	orf19.3895			
Cht3	orf19.7586			
Cht4	orf19.1515			
Eng1	orf19.3066	Endo-1,3-β-glucanase	Eng	GH81
Orf19.3417	orf19.3417			
Xog1/Exg1	orf19.2990	Exo-1,3-β-glucanase	Exg	GH5
Exg2	orf19.2952			
Spr1	orf19.2237			
Dfg5	orf19.2075	Mannosidase-like	Dfg	GH76
Dcw1	orf19.1989			

[a]According to classification at http://www.cazy.org. GH, glycoside hydrolase.

CELL WALL GLYCOPROTEINS

The cell wall is considered a crucial component of the fungal cell, but the majority of studies have focused on the synthesis and structure of the cell wall polysaccharides presented in the previous section. Nevertheless, proteins are known to represent a large part of the cell wall (around 35%), but their functions remained poorly understood. The main reason for this is that their extraction and purification are difficult because most are highly N and O glycosylated, and they are tightly linked to the other structural cell wall components. Fortunately, during the last decade, a number of different techniques have been developed to extract and study cell wall components of *Candida* species, including a combination of physical, chemical, and enzymatic methods, in order to break the cells, digest the covalent linkage, and partially remove the associated glycans. In addition, the availability of genome sequences coupled to progress in mass spectroscopy has considerably increased our knowledge of CWPs. Prior to the availability of genome sequences, CWPs, for example, MP65, were mostly described and named according to their molecular weight (32, 70). With the application of postgenomic approaches, computer-based algorithm predictions, and gene knockout technologies, the corresponding genes have been identified and characterized in detail. The main class of CWPs are covalently attached to the wall via a modified glycosylphosphatidylinositol (GPI) anchor. Sequence data coupled to prediction software have identified around 108 putative GPI-anchored proteins (2, 41, 48, 106, 178) in the *C. albicans* genome, whereas only around 31 have been detected by cell wall proteomics (30, 40, 125, 192). These proteins have been extensively reviewed (31, 95, 178), and in this section the main information is summarized and the latest developments described.

Subgroups of CWPs and Anchoring Processes

Proteins located in the cell wall can be classified according to the different treatments required to extract them. Some are released from the cell wall by sodium dodecyl sulfate (SDS) under reducing conditions and are therefore called noncovalent, soluble cell wall proteins. The majority, however, are covalently attached to the glucan layer and can be released from SDS-extracted cell walls by β-glucanases (87, 93, 178). There are two modes of covalent linkage: most cell wall proteins are tethered to the wall via a modified GPI anchor to β(1,6)-glucan or in a small minority via an alkali-labile bond to β(1,3)-glucan. Therefore, CWPs can be divided into three groups. (i) The vast majority are GPI-anchored proteins that are covalently linked to glucan. (ii) The Pir proteins (protein with internal repeats) are linked by a mild alkali-sensitive linkage to the cell wall (220). There are two Pir proteins in the *C. albicans* genome, Pir1 and Pir32, but only Pir1 has been clearly identified in cell wall extracts (130). (iii) Proteins that are extracted from the cell wall using SDS and/or a reducing agent like β-mercaptoethanol are commonly identified as cell wall localized but are also often detected in the surrounding medium; some have enzymatic function related to cell wall structure (6, 150, 185) or are cytoplasmic proteins associated with glycolysis, stress, and fermentation (69, 117, 164), and some have immunomodulating functions (128, 165, 185).

Soluble Cell Wall Proteins

The third group, composed of soluble cell wall proteins, can again be subdivided. Some, like enolase, alcohol dehydrogenase, pyruvate kinase, phosphoglycerate mutase, and methionine synthase, lack a signal peptide and may be exported by nonclassical export pathways (34). Others display a normal and functional signal peptide, like Pra1 and the β(1,3)-glycosyltransferase Bgl2 (128, 187). Interestingly, although Bgl2 proteins are not GPI anchored but are retained in the cell wall by some weak bonds, the cell needs a fully functional GPI-anchored synthesis pathway for correct incorporation of Bgl2 into the *S. cerevisiae* cell wall (85). This suggests that there is a strong interdependence between the different groups of CWPs, probably through the cell wall polysaccharides. A defect in GPI-anchored synthesis would affect GPI-anchored proteins, and since many of them have a role in cell wall assembly, this would affect cell wall structure, including associated proteins such as Bgl2.

Proteins associated with glycolysis, like enolase and alcohol dehydrogenase, are well-known cytoplasmic proteins but have also been identified at the cell surface; this dual location has led them to be termed "moonlighting" proteins. There is controversy surrounding the cell wall localization of these proteins, with some studies arguing that they are a contamination of cell wall samples, perhaps released from leaky or lysed cells during the extraction process (94), whereas others consider this second location genuine (156). The 169 N-terminal amino acids of enolase have been shown to be sufficient to target the protein to the cell surface, supporting the argument that enolase is genuinely surface localized (119). This debate will continue until a nonconventional secretion pathway is discovered.

GPI-Anchored Proteins

GPI anchoring is encountered in every eukaryotic cell, including unicellular yeast cells, several parasites, and highly specialized mammalian cells (48, 51, 81). It is the most common way to localize proteins to the cell surface in eukaryotes. The core GPI anchor consists of a lipid group (serving as the membrane anchor), a *myo*-inositol, an *N*-acetylglucosamine, three mannose sugars, and a phosphoethanolamine group, which ultimately connects the GPI anchor to the protein via an amide linkage. Shortly after protein synthesis, in the endoplasmic reticulum (ER), the preformed GPI anchor, also produced in the ER, replaces the C-terminal transmembrane region typical of all GPI-anchored protein sequences (198). Indeed, proteins destined to be GPI anchored share conserved features: an N-terminal signal sequence for localization to the ER, a C-terminal hydrophobic domain (9 to 24 residues long) for transient attachment to the ER membrane, and the so-called ω site, where the protein is cleaved and ligated to the GPI anchor (202). Kapteyn et al. showed that some GPI-anchored proteins in *S. cerevisiae* became covalently linked to cell wall β(1,6)-glucan after cleavage of the GPI anchor (88). In *S. cerevisiae*, this cleavage event occurs between the *N*-acetylglucosamine group and the first mannose of the anchor. The protein with the GPI anchor remnant is then transported to the cell wall and linked to β(1,6)-glucan by an unidentified protein or protein complex (123). Some GPI-anchored proteins remain attached to the plasma membrane, while others are translocated from the plasma membrane and become covalently linked in the cell wall in *S. cerevisiae* and *C. albicans* (56, 126, 127). Several studies have tried to define the key features determining the final destination of GPI-anchored proteins, either plasma membrane or cell wall, and have highlighted the residues around the ω site. Based on studies with *S. cerevisiae*, there are few specific sequence features that are conserved among GPI-anchored proteins. Two signals have been proposed for cellular localization in *S. cerevisiae*: (i) the specific amino acid residues V, I, or L at site ω-4

or ω-5, upstream of the GPI attachment site (the ω site) and Y or N at site ω-2 for cell wall localization and (ii) dibasic residues (R and K, for instance) in the region upstream of the ω site for plasma membrane localization (55, 72). Green fluorescent protein (GFP) fusions have been used as a reporter system in *C. albicans* to test key residues for their influence on protein localization in order to verify the predictions made from sequence analyses and to improve the criteria (127). The prediction concerning the ω site localization was confirmed by point mutation G613 in an Hwp1-GFP fusion and its substitution with permitted and nonpermitted residues (127). This experiment confirmed how little is known about the rules governing GPI anchoring, since some nonpermitted substitutions caused GFP to accumulate within the cell and others did not. In a second, more recent study, Mao et al. used two proteins with very distinct localizations: Hwp1 for the cell wall and Ecm331 for the membrane (126). They confirmed in *C. albicans*, as in other yeasts, the importance of the ω-5 to ω-1 amino acids for distinction between cell wall or plasma membrane (PM) targeting. Replacing amino acids from Hwp1 with amino acids from Ecm331 at this position clearly redirected the Hwp1-GFP fusion to the PM. However, the magnitude of the effect varied strongly depending on which amino acid was introduced at this position, demonstrating that signals other than ω-5 to ω-1 also contribute to the targeting. In conclusion, they refined the rules that can be used for prediction of localization: (i) I, V, or L residues at positions ω-3 to ω-8 or FE, FD, Y, or N residues at positions ω-1 to ω-3 are cell wall targeting signals and (ii) K or R residues at positions ω-1 to ω-7 or KK or SS dipeptides at positions ω-1 and ω-2 are PM retention signals. With this improved definition the prediction correlates well with the experimental data, with around a 15% error rate (126). Of the 104 predicted GPI-anchored protein sequences studied, 64 were predicted to be cell wall targeted and 22 PM targeted, confirming the predicted localization of the vast majority of the GPI-anchored proteins to the cell wall.

To date, thanks to advanced proteomic technologies, 31 GPI-anchored proteins have been identified in the cell wall (30, 31, 40, 95, 125, 192): Pga4, Pga10, Pga30, Pga31, Pga45, Pga59, Pga62, Als1, Als2, Als3, Als4, Cht2, Crh11, Csa1, Dfg5, Eap1, Ecm33, Hwp1, Phr1, Phr2, Pir1, Rhd3, Rbt1, Rbt5, Sap9, Sap10, Sod4, Sod5/Pga3, Ssr1, Utr2/Cfs4, and Ywp1. Furthermore, Als5, -6, -7, and -9 were localized in the cell wall based on heterologous expression in *S. cerevisiae* (191).

Pir Proteins

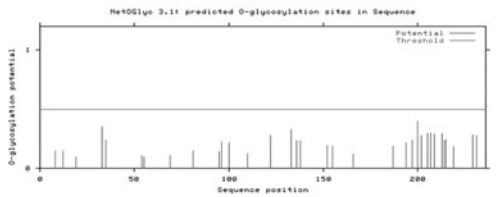
The second group of covalently linked CWPs, the Pir proteins, are attached to β(1,3)-glucans by alkali-labile bonds. In *S. cerevisiae*, the Pir proteins comprise Ccw5-8 (141, 221), and now a fifth Pir5 protein has been identified (see below). The extracting method first suggested that they were retained through an O-glycosidic linkage (87, 93, 136, 137, 142), but it has now been described as a covalent bond between a gamma-glutamine residue and β(1,3)-glucan. The bond is defined as an alkali-labile ester linkage between the carboxyl group of glutamic acid, arising from a specific glutamine, with a hydroxyl group from glucose of β(1,3)-glucan (46). In *S. cerevisiae*, localization experiments showed that Pir proteins are tethered to the cell wall but can be partly secreted, which is the case for Pir2 (184), or retained by several disulfide bonds, like Pir4 (138). Targeted deletion analysis of Pir proteins showed that the repeating sequence is absolutely required for binding to glucan, and mutagenesis experiments revealed that three glutamines and one aspartic acid within the sequence were essential. Glutamine at position 74 was identified as the linkage site (46). It is not known how this reaction takes place and which enzyme is responsible for the formation of the ester linkage (200). There is also the possibility of autocatalytic incorporation of these proteins into the cell wall. In *C. albicans*, studies using *Sc*Pir antibodies found two Pir-immunoreactive bands in cell wall fractions from cells grown in synthetic complete medium but not when cells were grown in RPMI medium (87). These two bands may represent two alleles of Pir1 (orf19.220 and orf19.7851, 39 amino acids [aa] longer) or two different Pir proteins. Contrary to the case in *S. cerevisiae*, Pir proteins in *C. albicans* are linked only via the alkali-labile bond; disulfide bonds are not involved (130).

Structural Variations in CWPs

Most described cell wall proteins share a common domain organization: (i) an N-terminal domain that may harbor the functional domain, for example, the transglutaminase substrate domain of Hwp1 or the enzymatic domain in Cht2, and seldom contains potential N-glycosylation sites; (ii) a central domain of variable size, from 1,708 aa in Als3 to less than 50 aa in Pga59, with a high percentage of serine and/or threonine residues, as high as 53% in Hwp1, with a high probability of O glycosylation; and (iii) a C-terminal domain of various size traditionally thought to have low or no glycosylation (see below). This proposed archetypal structure of the CWPs is found in more than 75% (81 of 108 CWPs) of the total putative CWPs, which agrees with the cell wall prediction figures obtained by analyzing the sequence environment of the ω site (see previous section).

Analysis of the O glycosylation predicted patterns of the entire GPI-anchored protein group using the website of the Danish Center for Biological Sequence Analysis in Lyngby (http://www.cbs.dtu.dk/services/NetOGlyc/) revealed four spatial organizations (Fig. 2).

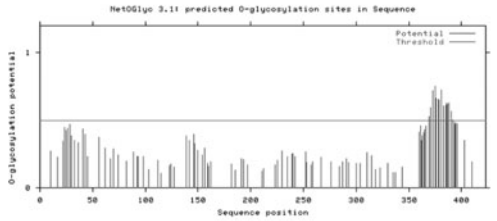
- Group 1 comprises 27 proteins that have no O-glycosylation sites either because there is no residue for glycosylation or because the sites have a probability of glycosylation lower than the calculated threshold. Comparison between this list and the proteins experimentally localized in the cell wall confirms the hypothesis that the domain subdivisions described above are characteristic of true CWPs. Indeed, of these 27 proteins, only 2 have been experimentally identified in the cell wall: Pga45 (30), which has the correct signal in the ω-5 to ω-1 domain (see above), and Dfg5 (194), which was demonstrated to be both PM and cell wall localized, with the majority present in the PM. This suggests that the 25 remaining proteins are very likely to be PM proteins. Confirming this hypothesis, the few known proteins of this group are two phospholipases (Plb), likely to function at the PM (Plb3 and Plb4); Exg2, a putative exo-β(1,3)-glucosidase experimentally located at the PM (22); Spr1, a member of the Exg2 family; Mid1, a calcium channel (16); and, finally Iff7, which has been suggested from sequence analysis to be anchored in the PM by a transmembrane domain rather than a GPI anchor (15).
- Group 2 is made up of 23 proteins that are virtually free of O glycosylation on the majority of the protein length but have a short C-terminal domain that is clearly O glycosylated (Fig. 2). More than half of



Pga43

Pga1*, Pga5/Gas2, Pga9/Sod6, Pga11*, Pga14*, Pga15*, Pga16, Pga17, Pga19, Pga27, Pga29/Rhd3*, Pga45, Pga46*, Pga48, Pga49, Pga51/Dcw1, Pga52, Pga56, Crh12, Dfg5, Exg2, Iff7, Mid1, Plb3, Plb4, Spr1

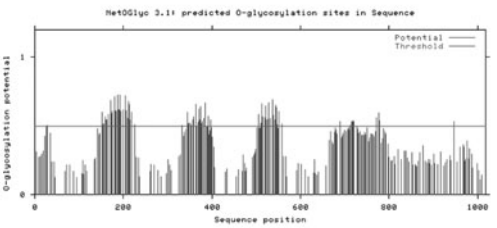
27 proteins



Ecm33

Pga2/Sod4, Pga3/Sod5, Pga4/Gas1, Pga7/Crw3, Pga10, Pga21, Pga28, Pga30, Pga31, Pga50, Pga63, orf19.4955, Cht1, Cht2, Crh11, Ecm331, Phr1, Phr2, Plb5, Sap9, Sap10, Utr2

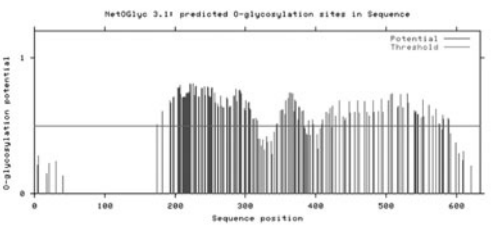
23 proteins



Csa1

Pga34, Pga37,Pga38, Pga57, Pga60, Pir1, Pir32

8 proteins



Hwp1

Pga6, Pga8/Hwp2, Pga13, Pga18, Pga20, Pga22, Pga23, Pga24/Ywp1, Pga25, Pga26, Pga32, Pga33, Pga35/Frg41, Pga36/Ihd1, Pga39, Pga40/Frg23, Pga41, Pga42, Pga44, Pga47/Eap1, Pga53, Pga54, Pga55, Pga58, Pga59, Pga61, Pga62, orf19.4652, orf19.4653, Als1, Als2, Als3, Als4, Als5, Als6, Als7, Als9, Hyr1, Iff2, Iff3, Iff4, Iff5, Iff6, Iff8, Iff9, Rbt1, Rbt5, Ssr1, Kre1

50 proteins

FIGURE 2 Analysis of the predicted O-glycosylation patterns of C. *albicans* cell surface glycoproteins using the NetOGlyc 3.1 Server of the Danish Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/NetOGlyc/). One representative pattern is used to illustrate the four different categories identified. Asterisks indicate the proteins of group 1 with neither O glycosylation nor N glycosylation predicted. 10.1128/9781555817176.ch14f2

these proteins have a described putative function. Included are the aspartyl proteases/yapsins (Sap9 and -10), chitinases (Cht1 and Cht2), superoxide dismutases (Sods; Sod4 and Sod5), a Plb (Plb5), and six carbohydrate-active enzymes involved in the remodeling of glucan; three putative glucanosyltransferases (Phr1, Phr2, and Gas1); and three glycoside hydrolases (Ecm331, Utr2, and Crh11) (Tables 1 and 2). So the majority of the GPI-anchored enzymes that have roles in cell wall remodeling have comparable O-glycosylation organizations with a short O-glycosylated C-terminal domain. One hypothesis is that this small region increases the cell wall linkage efficiency or participates in the recognition required for cell wall anchorage. Indeed, 11 of the 21 proteins have been experimentally located to the cell wall (see above).

- The third and fourth groups are composed of proteins that are heavily glycosylated (Fig. 2). The smaller group, composed of only eight proteins, has an unusual pattern of O glycosylation, with a series of highly and poorly glycosylated domains succeeding each other. Surprisingly, this pattern of glycosylation is not related to sequence tandem repeats (see section below). The best example is illustrated with Csa1 (Fig. 2), with four successions or Pga38 with five oscillations, but in some cases it may only be two successions, like in Pga60. It would be easy to speculate that such a repetitive pattern of glycosylation may be of importance for the immunogenic properties of these proteins, since Csa1 was first described as an antigen with strong immunogenicity (105). Alternatively, this structural organization might also increase adherence properties; this particular issue remains to be addressed experimentally.
- The fourth group is composed of 50 proteins that have the archetypal CWP structure with a large middle domain that is heavily glycosylated. Interestingly, most of the well-known and experimentally

TABLE 2 *Candida* species predicted GPI-anchored protein families

	Predicted GPI-anchored protein families in *Candida* species[f]						
Family	*C. albicans*	*C. dubliniensis*	*C. tropicalis*	*C. parapsilosis*	*C. guilliermondii*	*C. lusitaniae*	*C. glabrata*
Family 1, chitinase							
Cht1	orf19.7517	Cd36_25200	CTRG_01065	CPAG_04350		CLUG_05786	
Cht2	orf19.3895	Cd36_53830	CTRG_05456	CPAG_05456 CPAG_05457 CPAG_05454			
Cht3	*orf19.7586*	*Cd36_35130*	*CTRG_05827*	*CPAG_01567*	*PGUG_02893*		*CAGL0M09779g*
Cht4[a]	*orf19.1515*	*Cd36_16810*	*CTRG_01427*	*CPAG_01022*	*PGUG_02718*	*CLUG_00319*	
Family 2, DFG-like							
Dfg5	orf19.2075	Cd36_15490	CTRG_01122	CPAG_04572	PGUG_02704	CLUG_00324	CAGL0M05049g
Dcw1	orf19.1989	Cd36_16220	CTRG_01228	CPAG_04639	PGUG_04956	CLUG_02888	CAGL0L01727g
Family 3, Ecm3-like							
Ecm33.1	orf19.4255	Cd36_52240	CTRG_06015	CPAG_02560	PGUG_01073	CLUG_01897	CAGL0E04620g
Ecm33.3	orf19.3 010.1	*Cd36_02980*				CLUG_03566	CAGL0M01826g
Ecm33-like	orf19.4955	*Cd36_12330*	*CTRG_03600*	*CPAG_05634*	PGUG_03185	CLUG_04818	CAGL0H01661g
Family 4, exoglucanases							
Exg1, Xog1	*orf19.2990*	*Cd36_02790*	*CTRG_04334*	*CPAG_04841*	*PGUG_04742*	*CLUG_04461*	*CAGL0I00484g*
Exg2	orf19.2952	Cd36_02450	*CTRG_04664*	CPAG_04904	PGUG_03981	*CLUG_01602*	CAGL0M08756g
Spr1	orf19.2237	Cd36_21140	*CTRG_01901*	*CPAG_02812*			
Family 5, Pga37/57							
Pga37	orf19.3923	Cd36_54030					
Pga57	orf19.4689	Cd36_41010					
Family 6[b]							
Pga59	orf19.2767	Cd36_42250	CTRG_00155	CPAG_03756			
Pga62	orf19.2765	Cd36_42270	CTRG_00154	CPAG_00861	PGUG_02520 PGUG_02521 *PGUG_04576*		CAGL0M11726g CAGL0L06424g
Family 7, yapsins[c]							
Sap9	orf19.6928	Cd36_83850	CTRG_02621	CPAG_01798	PGUG_01925 PGUG_00002	CLUG_03179 CLUG_04902 CLUG_04189 CLUG_05007	CAGL0M04191g CAGL0E01419g CAGL0E01771g

(Continued on next page)

TABLE 2 *Candida* species predicted GPI-anchored protein families *(Continued)*

	Predicted GPI-anchored protein families in *Candida* species[f]						
Family	*C. albicans*	*C. dubliniensis*	*C. tropicalis*	*C. parapsilosis*	*C. guilliermondii*	*C. lusitaniae*	*C. glabrata*
						CLUG_05008	
						CLUG_05009	
						CLUG_05239	
						CLUG_05450	
						CLUG_05534	
						CLUG_05592	
						CLUG_03227	
						CLUG_03228	
						CLUG_03229	
Sap10	orf19.3839	Cd36_44180	CTRG_00096	CPAG_05341		CLUG_00903	CAGL0E01793g
			CTRG_02698	CPAG_01800			CAGL0E01837g
			CTRG_02710	CPAG_02614			CAGL0E01815g
			CTRG_02711	CPAG_04721			CAGL0E01859g
			CTRG_03508	CPAG_04722			CAGL0E01727g
			CTRG_00264	CPAG_01799			CAGL0A02431g
				CPAG_02617			
				CPAG_00343			
				CPAG_00659			
Yps7	*orf19.6481*	*Cd36_72090*	CTRG_05014	CPAG_04713	*PGUG_04882*	CLUG_03056	
						CLUG_04798 (Sap3 ortholog)	
Family 8, Crh-like							
Crh12	*orf19.3966*	*Cd36_54410*	*CTRG_06095*	*CPAG_01977*	*PGUG_03025*	*CLUG_01810*	*CAGL0J08910g*
					PGUG_02516		
Utr2	orf19.1671	Cd36_81610	CTRG_02140	CPAG_03890	PGUG_00573	CLUG_02005	CAGL0C02211g
Crh11	orf19.2706	Cd36_42770	CTRG_00263	CPAG_03194	PGUG_02387	CLUG_04980	CAGL0G09449g
Family 9, Sod-like[e]							
Pga2, Sod4	orf19.2062	Cd36_15610	CTRG_01132	CPAG_04555	PGUG_00849		
Pga3, Sod5	orf19.2060	Cd36_15620				*CLUG_03427*	
Pga9, Sod6	orf19.2108	Cd36_15220	CTRG_01087	CPAG_04598	*PGUG_02053*	*CLUG_0792*	
					PGUG_03463		
Family 10							
Pga15	orf19.2878	Cd36_65070					
Pga41	orf19.2906	Cd36_45820					
Pga42	orf19.2907	Cd36_45820					

Family 11[d]							
Pga28	orf19.5144	Cd36_72760	CTRG_04995	CPAG_04352			
			CTRG_04996	CPAG_00175			
			CTRG_04968				
			CTRG_04969				
			CTRG_04970				
			CTRG_04988				
			CTRG_04989				
			CTRG_03992				
"Pga36, Idh1"	orf19.5760	Cd36_64370	CTRG_02718				
			CTRG_05420				
Pga50	orf19.1824						
Pga61	orf19.5762	Cd36_64390	CTRG_05421				
			CTRG_05422				
			CTRG_04054				
orf19.7130	orf19.7130	Cd36_73980	CTRG_04971	CPAG_03490			
Family 12							
Hwp1	orf19.1321	Cd36_43360					
Rbt1	orf19.1327	Cd36_43400	CTRG_00477	CPAG_00831			
			CTRG_00478	CPAG_02691			
Pga8, Hwp2	orf19.3380	Cd36_43420					
Family 13[c]							
Pga29/Rhd3	orf19.5305	Cd36_43810	CTRG_00299			CLUG_02844	
Pga30	orf19.5303	Cd36_43790	CTRG_00349	CPAG_04045	PGUG_01942		
			CTRG_00352	CPAG_04046			
Pga31	orf19.5302	Cd36_43780	CTRG_00348				
			CTRG_00350				
Family 14[c]							
Plb3	orf19.6594	Cd36_34860	CTRG_05889	CPAG_04322		CLUG_01525	CAGL0J11770g
			CTRG_01633	CPAG_04323			
Plb4	orf19.1442	Cd36_16240	CTRG_01230		*PGUG_02695*	CLUG_02215	CAGL0J11748g
Plb5	orf19.5102	Cd36_07760	CTRG_03153	CPAG_02207	PGUG_01288	CLUG_00164	CAGL0E02321g
				CPAG_02060			
Family 15[e]							
Csa1, Wap1	orf19.7114	Cd36_70300	CTRG_04939	CPAG_00621		CLUG_04093	
			CTRG_03125	CPAG_00622			
			CTRG_04948				

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TABLE 2 *Candida* species predicted GPI-anchored protein families *(Continued)*

	Predicted GPI-anchored protein families in *Candida* species[f]						
Family	*C. albicans*	*C. dubliniensis*	*C. tropicalis*	*C. parapsilosis*	*C. guilliermondii*	*C. lusitaniae*	*C. glabrata*
Pga7	orf19.5635	Cd36_40180	CTRG_00098	CPAG_02388		CLUG_04096	
			CTRG_00108	CPAG_02389			
Rbt8, Rbt51	orf19.5674	Cd36_40510					
Rbt5	orf19.5636	Cd36_40190	CTRG_00099	CPAG_02386		CLUG_04097	
			CTRG_00109	CPAG_02387			
Csa2	*orf19.3117*	*Cd36_46390*					
Rbt5-like				*CPAG_05259*			
Family 16							
Pga4	orf19.4035	Cd36_54990	CTRG_06145	CPAG_02503	PGUG_00517	CLUG_04859	*CAGL0F01287g*
						CLUG_02142	
Pga5	orf19.3693	Cd36_02200	CTRG_04635	CPAG_04876			CAGL0G01056g
Phr1	orf19.3829	Cd36_44230	CTRG_03942	CPAG_01721	PGUG_05875	CLUG_01043	
Phr2	orf19.6081	Cd36_00220	CTRG_04296	CPAG_00279	PGUG_02099	*CLUG_00398*	CAGL0M13849g
						CLUG_00399	
Phr3	*orf19.377*	*Cd36_40150*	*CTRG_00112*	*CPAG_04070*	*PGUG_01388*	*CLUG_04858*	CAGL0G00286g
Gas3							CAGL0F03883g
Gas4							CAGL0E01595g
Family 17			No orthologues defined in other species; can only define as Als-like				For all adhesin like sequences of *C. glabrata*, see reference 42
Als1	orf19.5741	Cd36_64210	*CTRG_02293*	CPAG_00368	PGUG_03330	CLUG_03274	
Als2	orf19.1097	Cd36_65010	*CTRG_03786*	CPAG_05056	*PGUG_02302*		
		Cd36_64800	*CTRG_01028*	CPAG_05314	PGUG_03259		
Als3	orf19.1816		CTRG_03791	CPAG_00369	PGUG_00673		
Als4	orf19.4555	Cd36_64610	*CTRG_03797*	CPAG_05054			
Als5	orf19.5736		CTRG_02229				
Als6	orf19.7414	Cd36_86290	*CTRG_02228*				
Als7	orf19.7400	Cd36_86150	CTRG_01030				
Als9	orf19.5742	Cd36_64220	CTRG_00941				
			CTRG_03787				
			CTRG_03882				
			CTRG_03871				
			CTRG_01041				
			CTRG_01038				

Family 18			No orthologues defined in other species; can only define as Iff-like				
Hyr1	orf19.4975		CTRG_00233	CPAG_01112	PGUG_01202	CLUG_04844	
Iff1	orf19.5124	Cd36_72940	CTRG_01921	CPAG_01114	PGUG_01219	CLUG_05233	
Iff2	orf19.575	Cd36_51670	CTRG_01923	CPAG_01377	PGUG_02868	CLUG_05599	
Iff3	orf19.4361	Cd36_28830	CTRG_01926	CPAG_03917	PGUG_05906	CLUG_05603	
Iff4	orf19.7472	Cd36_25710	CTRG_02071	CPAG_03919	*PGUG_01214*	CLUG_05607	
Iff5	orf19.2879	Cd36_46030	CTRG_02188	CPAG_03920		*CLUG_01204*	
Iff6	orf19.4072	Cd36_23280	CTRG_02201	CPAG_04936		*CLUG_05593*	
Iff7	orf19.3279	Cd36_25840	CTRG_04426	*CPAG_00015*		*CLUG_05597*	
Iff8	orf19.570	Cd36_50710	CTRG_05838	*CPAG_00030*		*CLUG_05601*	
Iff9	orf19.465	Cd36_29150	*CTRG_00291*	*CPAG_01116*		*CLUG_05606*	
Iff10	*orf19.5404*	*Cd36_80540*	*CTRG_01922*	*CPAG_01176*			
Iff11	*orf19.5399*	*Cd36_80550*	*CTRG_01924*	*CPAG_01177*			
			CTRG_04224	*CPAG_03918*			
			CTRG_04685	*CPAG_03921*			
			CTRG_05066	*CPAG_04728*			
			CTRG_05198	*CPAG_04729*			
			CTRG_05200	*CPAG_04934*			
			CTRG_06222				
Family 19							
Pga52	orf19.1911	Cd36_15090	CTRG_01100	CPAG_04612	PGUG_04917	CLUG_00831	
Tos1	*orf19.1690*	*Cd36_81470*	*CTRG_02154*	*CPAG_03549*	*PGUG_00559*	*CLUG_01985*	
Family 20							
Kre1	orf19.4377	Cd36_29000	*CTRG_00718*	CPAG_05645	PGUG_00351	CLUG_05652	CAGL0M04169g
Pga1	orf19.7625	Cd36_35332	CTRG_05806	CPAG_01539	PGUG_05717	CLUG_03484	
Family 21					PGUG_04856	CLUG_01197	
ScMuc1-like						CLUG_05234	
						CLUG_05230	
Family 22						CLUG_04850[g]	
Similarity with N terminus of ScFlo9						CLUG_02906[g]	
						CLUG_05231[g]	
						CLUG_02909	
						CLUG_02910	

(Continued on next page)

TABLE 2 *Candida* species predicted GPI-anchored protein families *(Continued)*

Family	Predicted GPI-anchored protein families in *Candida* species[f]						
	C. albicans	*C. dubliniensis*	*C. tropicalis*	*C. parapsilosis*	*C. guilliermondii*	*C. lusitaniae*	*C. glabrata*
Family 23							
orf19.4652	orf19.4652	Cd36_41340					
orf19.4653	orf19.4653						

[a]Cht4 is more similar to bacterial chitinase and has low sequence similarity to Chtr1-3.
[b]Pga6 and its orthologues have some resemblance to other family 6 members and contain conserved YTTYCPL motifs.
[c]Only the GPI-anchored members of these large families are included here, but the complete family is illustrated in Fig. 4.
[d]Family 11 members have 8 cysteine residues with conserved spacing resembling so-called CFEM domains.
[e]Family 15 members have a putative CFEM domain according to the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml).
[f]Text in italics indicates no GPI anchor attachment site predicted.
[g]Contains a potential PA14 domain.

confirmed cell wall-localized adhesins are part of this group, including the Als (agglutinin-like sequence) family, Hwp1 and Hwp2, Eap1, Rbt1, and Rbt5. This confirms that part of the adherence properties developed by these proteins is due to the physicochemical properties of the central highly glycosylated domain (115, 223).

Pattern and Repeats

Analyses of the C. *albicans* and C. *dubliniensis* genomes to identify intragenic tandem repeats found a significant enrichment of putative CWPs (M. Legendre, C. A. Munro, and K. J. Verstrepen, unpublished data). Variation of tandem repeat numbers and sequence variability within isolates have the potential to greatly modulate cell wall antigenic properties (152). Verstrepen and Fink have described the surprisingly common principles of cell surface variability in evolutionary divergent microbes such as C. *albicans*, S. *cerevisiae*, and protozoa (208). Eukaryotic microorganisms have evolved ingenious mechanisms to provide cell surface variability using similar recombination mechanisms to promote the constant generation of novel genes using old genes as molecular building blocks. Variation in the repeat number of the *S. cerevisiae* flocculin Flo1 can significantly alter the physical properties of the yeast cell surface, including cell-to-cell adhesion (209). Several C. *albicans* CWPs are highly variable, for example, Als7 (222), and there is evidence of allelic differences as well as interisolate variation in terms of different numbers of tandem repeats (reviewed in reference 77).

CWPs that harbor intragenic tandem repeats can be divided into at least five different groups (Fig. 3).

1. The Als protein family is made up of eight GPI-anchored proteins (77). The Als proteins possess numerous copies of a tandem repeat sequence and are heavily N and O glycosylated. The Als repeats are conserved and 36 aa residues long, with cell-cell aggregation activities (175) (Fig. 3). These tandem repeats, localized in the central domain of each Als protein, can be used to classify the Als proteins into three subgroups according to their sequence similarity: (i) Als1 to Als4, (ii) Als5 to Als7, and (iii) Als9 (76). Specific analyses of these tandem repeat sequences revealed very large variation in the number of repeats between alleles of the same gene or between different clinical isolates. For example, the number of Als1 repeats ranged from 4 to 37; hence, these proteins may have participated in the evolutionary modification of this fungal pathogen (121, 124, 224). These changes in size correlate also, in the case of Als3, with differences in functionality, like adhesion to endothelial and epithelial cells (157). Structural modeling of the Als repeats suggest that there is structural conservation within the family and that the repeats form β-sheet domains with hydrophobic surfaces surrounded by hydrophilic glycans (54).

Another interesting feature of the Als family, initially described for Als5, is their ability to aggegrate in solution as amyloid-like fibers (159). Comparison of the protein sequences of the well-described adhesins in C. *albicans* and S. *cerevisiae* identified conserved patterns with beta-branched aliphatic amino acids Ile, Thr, and Val (174). These repeats were enriched in all the adhesins studied, and all, if purified, formed amyloid-like fibers. Thus, amyloid formation appears to be an intrinsic property of yeast cell adhesion proteins from many gene families and an important component of cellular aggregation mediated by them (174).

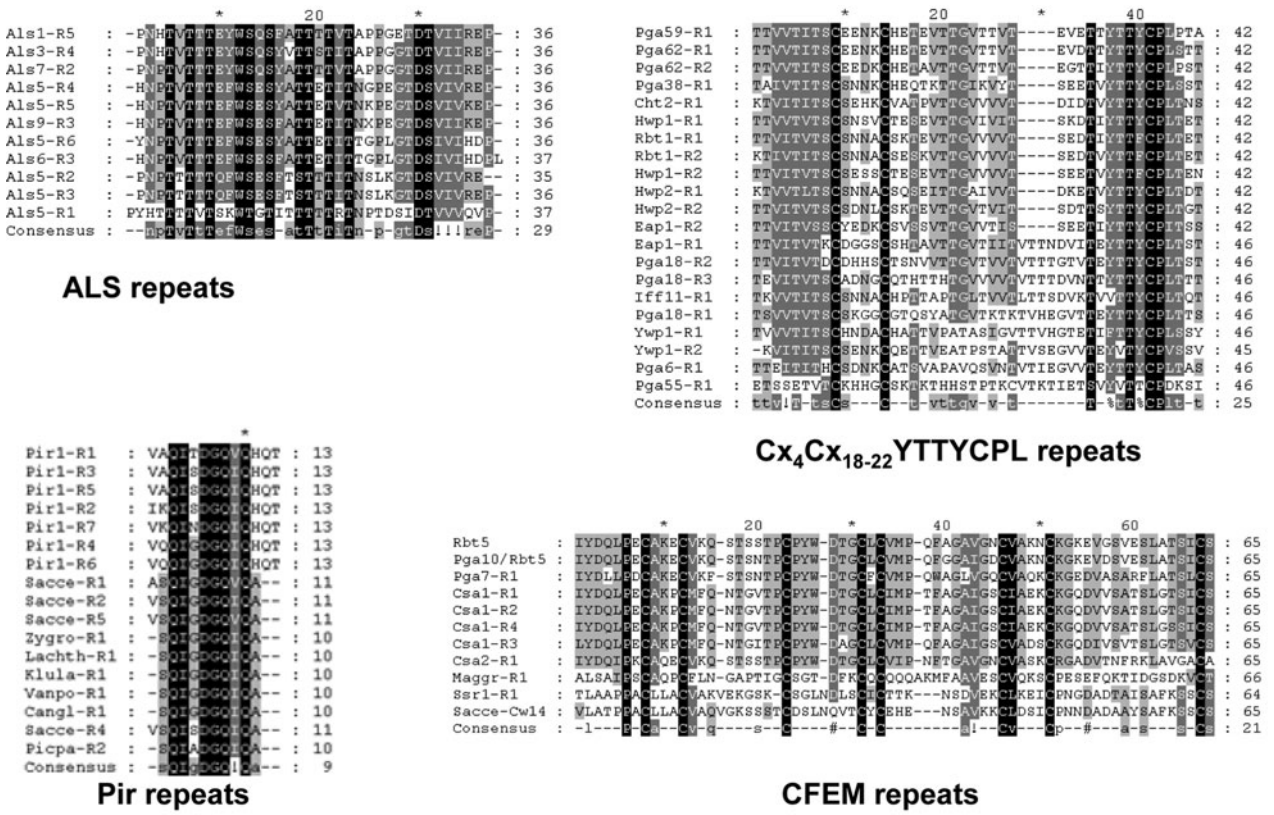

FIGURE 3 Tandem repeats identified in *C. albicans* cell surface glycoproteins. Cell surface glycoproteins can be classified according to sequence conservation within their intragenic tandem repeats. Four major groups harboring tandem repeats that share common features have been identified: Als, Pir, CFEM, and $Cx_4Cx_{18\text{-}22}$YTTYCPL. Representative repeats extracted from the *Candida* Genome Database (http://www.candidagenome.org/) are shown for the different proteins in each group. Additional sequences from other fungi have been added to some alignments to show the strong conservation between different fungal species. Maggr, *Magnaporthe grisea* G-coupled receptor Mac1; Sacce, *S. cerevisiae*; Zygro, *Zygosaccharomyces rouxii*; Lachth, *Lachancea thermotolerans*; Klula, *Kluveromyces lactis*; Vanpo, *Vanderwaltozyma polyspora*; Picpa, *Pichia pastoris*; Cangl, *C. glabrata*. Adapted from references 51, 99, and 129. 10.1128/9781555817176.ch14f3

2. Pir proteins were first described for *S. cerevisiae* (203). The four different Pir proteins (Pir1, Pir2/Hsp150/Ccw7, Pir3, and Pir4/Ccw5) are important for cell wall structure and for growth at low pH and in the presence of various inhibitors (calcofluor white [CFW], SDS, and amphotericin B) (133). A fifth protein, YJL160C, has been tentatively called Pir5. The common features shared by the *S. cerevisiae* Pir proteins are that (i) there is a consensus domain repeated twice in Pir4/Ccw5 and ten times in Pir2/Ccw7 (46) and (ii) they are all processed by the Kex2 protease. The canonical sequence found in five different proteins in *S. cerevisiae* is 12 aa long: SQIG**D**G**Q**V/I**Q**ATT/S. The three glutamines and the aspartic acid (in bold) are essential for binding to β(1,3)-glucan, and the glutamine at position 74 (underlined) is the linkage site. A similar sequence with some divergence can be found in Pir1 of *C. albicans* (VA**Q**IG**D**G**Q**-I**Q**HQT) but not in *Ca*Pir32 or in *Yl*Pir1 from *Yarrowia lipolytica* (84).

Sequence comparison of *S. cerevisiae* and *C. albicans* Pir proteins shows that most similarities are concentrated in the last 80 aa, a portion of the sequence with seven glutamine residues. A similar C-terminal domain is found in the *Y. lipolytica* Pir-like protein. Thus, even if the glutamine residue used for the ester linkage, described by Ecker et al., is the G74 in the tandem repeats, it is interesting to see that the C-terminal domain common to all Pir proteins is also rich in glutamine, with five to seven residues (46). One can hypothesize that in some situations one of these glutamines could be the linking site instead of the glutamine in the tandem repeats, at least in weakly related Pir proteins like the one in *Y. lipolytica* (84). Comparison of the Pir proteins in *S. cerevisiae* and *C. albicans* reveals that contrary to *Sc*Pir proteins, only *Ca*Pir1 has a putative site for Kex2 protease processing (between residues 18 and 19), and not *Ca*Pir32. Attempts to generate a *pir1*Δ mutant failed, suggesting that *PIR1* is essential (130). However, *pir1/PIR1* heterozygote strains showed defects in cell wall assembly, confirming that cell wall localization was in direct relation with its function (130) and that this protein seemed to share a comparable function with *Sc*Pir proteins. The specific biochemical function of the Pir proteins and the involvement of the tandem repeats in the functionality of the proteins remain unresolved.

3. Proteins with CFEM domains (for "common in several fungal extracellular membranes") have a 60-residue-long domain that consists predominantly of hydrophobic

residues and 8 conserved cysteines (Fig. 3). CFEM domains are present mainly in fungal and in some plant transmembrane or GPI-anchored proteins (103). *Magnaporthe grisea*, the causal agent of rice blast disease, contains 12 proteins with this domain, while only one CFEM protein is found in *S. cerevisiae* (Cw14) (104). In *C. albicans*, depending on the prediction algorithm, the number of CFEM-containing proteins varies from three to six and includes Rbt5, Rbt51/Pga10, Csa1, Csa2, Ssr1, and Pga7 (161, 213) (SMART prediction; http://smart.embl-heidelberg.de/). Csa2 is the only protein in this group that is not predicted to be GPI anchored; instead, it has a 29-aa C-terminal transmembrane domain, so altogether these proteins are likely to be surface localized in *C. albicans*. No function so far has been linked to this particular domain in *M. grisea* or in other fungi, but two laboratories studied the mutant phenotypes of CFEM proteins in *C. albicans*. Single, double, and triple mutants of *RBT5*, *RBT51*, and *CSA1* have been constructed (17, 161, 213). Rbt51 alone was sufficient to confer the ability to utilize hemoglobin iron when expressed in *S. cerevisiae*, and Rbt5 played the dominant role in heme-iron utilization in vitro (213). Deletion of *RBT51* has also been shown to result in sensitivity to Congo red, SDS, and CFW (161). In addition, *rbt5*Δ, *rbt5*Δ *rbt51*Δ, and triple *rbt5*Δ *rbt51*Δ *csa1*Δ mutants exhibited a thin and weak biofilm structure (161). Therefore, proteins with CFEM domains appear to play roles that contribute to *C. albicans* pathobiology.

4. Proteins with $Cx_4Cx_{18\text{-}22}$YTTYCPL tandem repeats. An interesting new pattern has been highlighted by Moreno-Ruiz et al. (135); this sequence is 42 to 46 aa long, with two conserved cysteines and a seven-residue domain, YTTYCPL (Fig. 3). The tandem repeats are found in 13 different proteins, with a total of 21 repetitions: Pga6, Pga18, Pga38, Pga55, Pga59, Pga62, Cht2, Eap1, Hwp1, Hwp2, Iff11, Rbt1, and Ywp1. All these proteins are putative GPI-anchored proteins, with 55% of them experimentally localized to the cell wall. An identical search of the *S. cerevisiae* database gives no hits even with the seven-residue sequence. Surprisingly, by reducing the length of the motif to TTYCP, two proteins, Sed1 and Spi1, are selected with three hits, and both of them are GPI-anchored proteins but have no other similarities to *C. albicans* proteins. These cysteine-containing motifs have been suggested to be responsible either for cell wall polysaccharide interaction, like the Gas1 domain, or for reinforcing the cell wall through intermolecular disulfide bridge formation (135). A detailed analysis of this tandem repeat is required to determine whether it plays a role in protein-protein interactions, such as homomultimers or heteromultimers, for example. Indeed, very little is known for *C. albicans* or other fungi about protein complexes at the cell surface or in the cell wall network.

5. Proteins with miscellaneous repeats. Among the cell surface glycoproteins, several have internal repeats that are specific to a single protein. Usually these repeats are small, as in Pga55 (114 repeats of SEVSSS), Pga18 (15 repeats of SATTPGSS), Eap1 (19 repeats of TESTPA), or Pga58 (6 repeats of PAPAPSA). In the case of the Eap1 repeats, a few related repeats showing small degrees of variation are found in Iff5 (one), Hwp1 (two), and Rbt1 (three), suggesting a possible role for this pattern in the adhesion process. Finally, some proteins have longer repeats much more difficult to identify due to small variations along the sequence, like Iff1 and -5. Thus, some members of the Iff family (see below) contain tandem repeats in their sequences, but the repeats do not have a conserved sequence or a common structure that is found in all Iff members. As there is a lack of conservation in these repeat sequences, it is difficult to assess any particular associated function. This is particularly true since the repeats are located in the central domain of the proteins, a domain often described with only a structural role: to present and carry the functional N terminus.

Families within the Surface Glycoproteins

The vast majority of cell surface proteins are represented by the GPI-anchored proteins, with around 108 putative proteins. The other groups are much smaller, with two Pir proteins and around a dozen soluble proteins. The soluble proteins do not fall into any particular gene families but belong to various biosynthetic pathways. Although the Pir proteins share a common pattern at their C termini, the overall similarity of the two sequences is rather low.

On the other hand, sequence comparison of the GPI-anchored glycoproteins has classified them into at least 23 families (21, 178) (Fig. 4, Table 2). Fourteen *C. albicans* families are small, composed of two or three proteins, while only three families have eight or more members: the SAP, ALS, and HYR/IFF families. Interestingly, only 10 *C. albicans* families are 100% composed of GPI-anchored proteins; the rest are mixed families composed of putative cell wall proteins and 27 proteins not predicted to be GPI anchored (Fig. 4). All of these 27 proteins have a predicted signal peptide for secretion, indicating that even if they are not GPI anchored, the localization of these proteins is the external environment of the cell. Additionally, only one GPI-anchored protein, Pga52, was found to have only a non-GPI-anchored protein homologue, Tos1 (Fig. 4). In this particular case, it is difficult to assess whether the GPI anchor prediction was wrong or whether the cells use two proteins with similar functions but distinct localizations, a situation observed 11 times with other mixed families.

Our limited understanding of the function or the regulation of these proteins hinders our abilities to interpret how *C. albicans* modulates its genome to end up with such diversity in localization among paralogous proteins. Nevertheless, it seems that the percentage of gene families is relatively high within GPI-anchored proteins, with a great deal of heterogeneous families composed of both proteins predicted to have GPI anchors and proteins that do not fall into this category.

ALS Family

One of the best-studied GPI-anchored protein families is the ALS family of adhesins, which is intensively described in chapter 7.

IFF Family

The largest family of GPI-anchored proteins, with 12 members, is encoded by the group of genes orthologous to *HYR1* (for "hyphally regulated"), also known as the *IFF* genes (for IPF [individual protein file] family F). There are no proteins in *S. cerevisiae* with significant similarity to any Iff family member. However, a large number of orthologues have been identified in the fungal species that are closely related to *C. albicans* forming the CTG clade (21). Although related, some of the orthologous genes are significantly divergent, and indeed, *Lodderomyces elongisporus* has hybrid genes that contain both Als-like and Iff-like elements (21), suggesting specific evolutionary divergence of these genes in this branch of the fungal phylogenetic tree. Within the *C. albicans IFF* family, 10 genes are predicted to have GPI anchors, and two genes, *IFF10* and *IFF11*, do not. However, the amino acid sequence of Iff10 is truncated compared to the

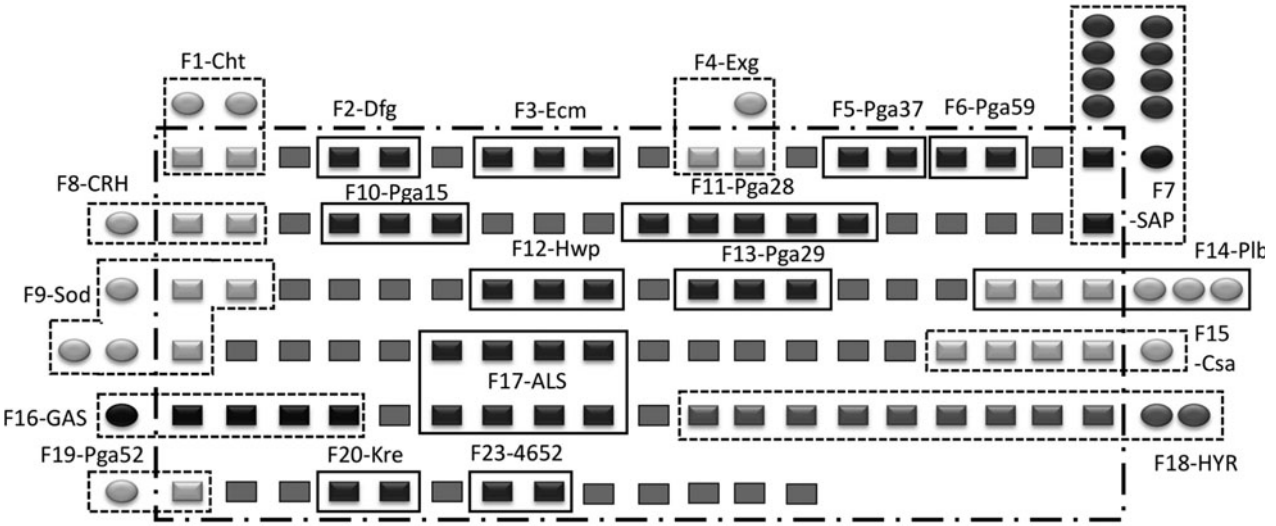

FIGURE 4 *C. albicans* glycoprotein families. The proteins are represented by a rectangle (predicted to be GPI anchored) or an oval (not predicted to be GPI anchored). The large group encircled by the dashed and dotted line represents the putative GPI-anchored proteins. Within this group there are 11 families of genes composed only of GPI-anchored proteins (surrounded by solid lines). In addition, there are 10 mixed families (marked with dotted lines) composed of one or more predicted GPI-anchored proteins and one or more non-GPI-anchored proteins. The families are labeled according to the nomenclature in Table 2. 10.1128/9781555817176.ch14f4

other Iff proteins and has no C-terminal region and thus no clear ω site for GPI anchoring. *IFF10* may be a pseudogene, or errors in the sequence may not have revealed the entire C terminus. In addition, as discussed earlier, Iff7 has a long hydrophobic tail which is closer to a transmembrane domain and may be membrane localized (15). Overall, the GPI anchoring and the cell surface localization of the Iff members are still to be confirmed by direct or indirect localization in vitro. Analysis of the coding sequences in this family revealed a large N-terminal domain of 340 aa shared by each member of the family with high homology (Fig. 5). The proteins diverge strongly after this domain, in both size and sequence. Phylogenetic analysis of this family shows that the two predicted non-GPI-anchored proteins, Iff10 and Iff11, diverge from the original group, but the others remain united (178). The functions of *HYR1*, *IFF4*, and *IFF11* have been studied by reverse genetics. The wide-ranging phenotypes of the three mutants do not suggest any conserved functions despite the large shared N-terminal domain. Hyr1 was first described as hyphally regulated under at least three different inducing conditions (alkaline and temperature switch, serum addition, and *N*-acetylglucosamine addition) (11, 12). Phenotypic characterization of the mutant did not provide any clues to Hyr1 function, nor did overexpressing the protein in *S. cerevisiae*. Germ tubes of the *iff4* mutant were less adherent to silicone and plastic, and the mutant had slightly decreased virulence in a mouse model of infection (90, 91). An *IFF4*-overexpressing strain was found to increase the adherence of *C. albicans* to plastic and to human epithelial cells but not endothelial cells (59). The overexpressing strain also had increased susceptibility to neutrophil-mediated killing and caused less severe hematogenously disseminated candidiasis in normal mice but not in neutropenic mice. These results suggest that Iff4 plays a role in adhesion and interaction with host cells, even if the mechanisms are yet unknown. Analysis of the *iff11*Δ mutant phenotype suggested an involvement of Iff11 in cell wall structure and in establishing an infection (15).

The Gas Glucanosyltransferase Family: Gas

The Gas family is composed of five members: Phr1, Phr2, Phr3, Gas1/Pga4, and Gas2/Pga5. These proteins, by homology with the *S. cerevisiae* Gas1 protein, are putative transglycosidases acting to remodel β(1,3)-glucan. This family is conserved in fungi (173); there are five Gas proteins in *S. cerevisiae* (170), three Gas proteins in *C. glabrata* (212), and at least two in *Aspergillus fumigatus* (73). They belong to the larger GH72 family of glycoside hydrolases in the carbohydrate-active-enzyme database (Table 1). Biochemical assays with heterologously expressed *Sc*Gas2, *Sc*Gas4, and *Sc*Gas4 in *Pichia pastoris* indicated that these three proteins also have β (1,3)-glucanosyltransferase activity similar to that of *Sc*Gas1 (173). A domain designated the Cys box found in only half of the Gas family members is required for disulfide bond formation and normal folding of Gas1 and Gas2 (169) (Fig. 5). Mutants of *GAS* genes in *C. albicans*, *S. cerevisiae*, *C. glabrata*, and *A. fumigatus* had defects in cell wall organization and morphogenesis. Single and double disruption of the *A. fumigatus* Gas orthologues *GEL1* and *GEL2* showed that the enzymatic activity was required for morphogenesis and virulence (140). Accordingly, in *C. albicans* the *phr1*Δ and *phr2*Δ mutants had reduced virulence in animal models, with different specificities depending on the model (vaginal or systemic) (38). The *pga4*Δ mutant has only been tested in an oral epithelial tissue infection model and had a wild-type phenotype (45).

The Crh Family of Transglycosidases

In *C. albicans*, the Crh family is encoded by three genes, *CRH11*, *CRH12*, and *UTR2*, related to their *S. cerevisiae* orthologues *ScCRH1*, *ScUTR2/CRH2*, and *ScCRR1*. In

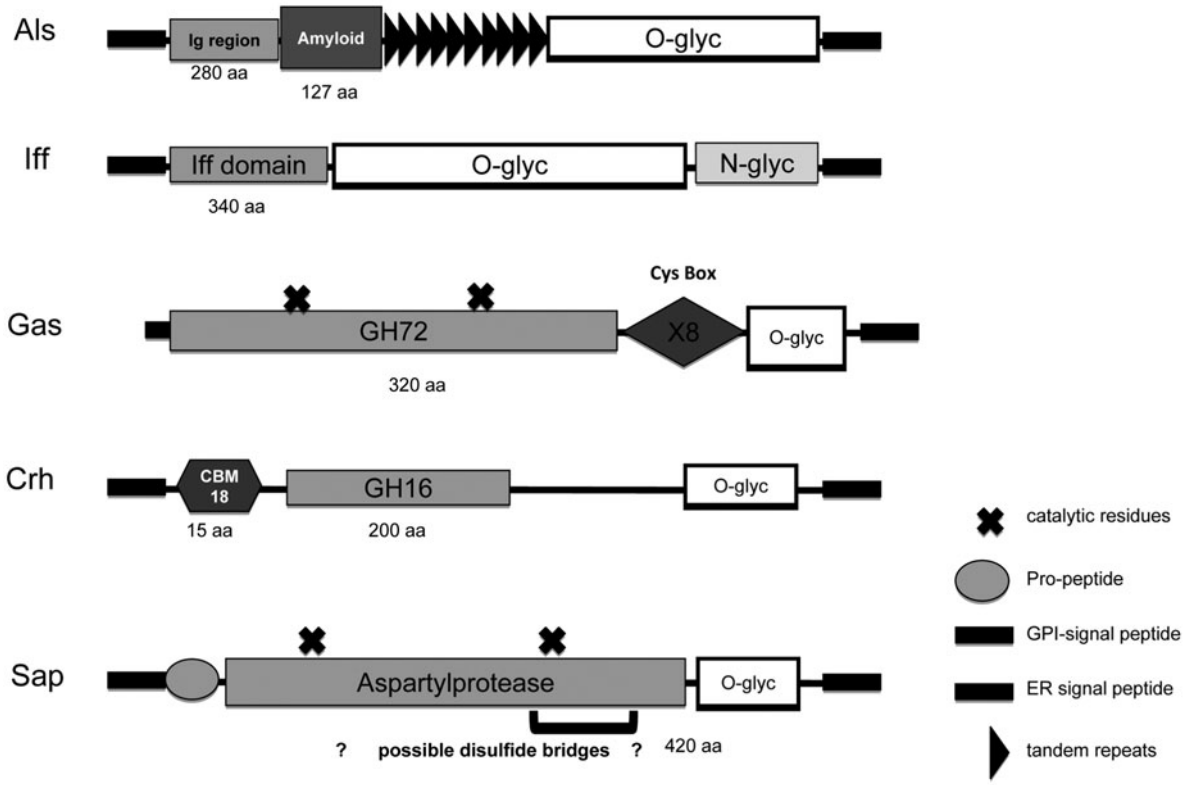

FIGURE 5 Domain organization of glycoprotein families of *C. albicans*. A common domain organization can be defined for each glycoprotein family. All families display a posttranslational modified domain that is O and/or N glycosylated. The black boxes at the N and the C termini are the signal peptide and the GPI anchor addition signal, respectively. Als proteins consist of tandem immunoglobulin (Ig)-like domains, a β-sheet-rich conserved 127-residue amyloid-forming T region, and a variable number of tandem repeats characteristic of the Als proteins. Iff proteins share a common N-terminal domain of 340 to 350 aa with no predicted specific function. Gas proteins share a GH72 domain (CaZY domain for β(1,3)-glucanosyltransglycosylase, EC 2.4.1) with two catalytic sites located on two glutamic acids at positions 152 and 254 for Pga4/Gas1. The Cys box is the cysteine-enriched module present in Phr1 and -2 and Pga5/Gas2 but not in Phr3 or Pga4/Gas1. Crh proteins share two functional domains, a GH16 domain (CaZY domain for endo-1,3-β-glucanase, EC 3.2.1.39), and a carbohydrate-binding module (CBM18) for binding chitin, but the last is present only in Utr2/Csf4 and not in Crh11 or Crh12. In the case of Sap proteins, after the propeptide, characteristic of this family, the proteins share an aspartyl protease catalytic domain with two aspartic acid catalytic residues at positions 83 and 380 in Sap9. Four cysteine residues may be responsible for the formation of two disulfide bridges within the catalytic domain. Adapted from references 2, 57, 153, and 166. 10.1128/9781555817176.ch14f5

S. cerevisiae, the Crh family is involved in cell wall construction (180). *Sc*Crh1 and *Sc*Crh2 are directly responsible for the attachment of chitin to both β(1,3)-glucan and β(1,6)-glucan (23, 25). These results fill a large gap in our comprehension of the mechanisms of cell wall assembly. Deletion of *ScCRH1* and *ScCRH2* results in additive sensitivity to Congo red and CFW that interferes with cell wall assembly. Moreover, the double deletion mutant showed a twofold increase in the amount of alkali-soluble cell wall glucan in comparison to that of the wild type, indicating that less glucan is bound to chitin (180). Tandem mass spectrometry analysis of the cell wall proteome showed that both proteins were covalently bound to the cell wall (220). GFP fusion proteins indicated that Crh1 and Crh2 are located in chitin-rich areas and that their temporal and spatial localization is specifically controlled during the cell cycle (181).

The Crh family is conserved in fungi and belongs to the larger GH16 family of glycoside hydrolases in the CAZy database (Fig. 5 and Table 1). The *C. albicans crh*Δ mutants have phenotypes similar to those of their *S. cerevisiae* counterparts. The *utr2*Δ mutant exhibited a reduced adherence to FaDu epithelial cells and a defect in cell wall integrity (2). Single, double, and triple mutants of the *Ca*Crh family were also hypersensitive to cell wall-perturbing agents, and the triple mutant was avirulent in a mouse model of systemic infection (160). In the triple mutant the cell wall defect was compensated for by increased chitin content, through the activation of, at least, the PKC salvage pathway, a well-defined remodeling pathway triggered under cell wall stress conditions (153). The *CaCRH* genes are the downstream targets of the Ca^{2+}/calcineurin pathway acting through the Crz1 transcription factor (89).

The Crh and the Gas families have immunogenic and antigenic properties. Proteins of these families reacted with sera from patients infected or not infected with *C. albicans* or *A. fumigatus* (9). Therefore, these families are confirmed cell surface-localized enzymes that contribute to cell wall assembly and remodeling in response to cell wall stress, are required for virulence, and are immunologically reactive.

The Sap Family

The *C. albicans* secreted aspartyl protease (Sap) family is composed of 10 proteins, Sap1 to Sap10. Two of them, Sap9 and Sap10, are predicted to be GPI anchored. In the *S. cerevisiae* cell wall, anchored aspartyl proteases, also termed yapsins, play important roles in cell wall assembly, acting as sheddases, proteolytically cleaving other CWPs. Known substrates include *Sc*Gas1 and yapsins themselves as well as the signaling mucin Msb2 (206). Orthologous roles are likely for *Ca*Sap9, as it can complement *S. cerevisiae yps1*Δ (102). There are numerous studies on the function and expression of the secreted Saps; see, for example, references 79, 80, 148, and 188. In this chapter, we focus on the predicted GPI-anchored proteins Sap9 and -10 (Fig. 5).

Yapsins in *S. cerevisiae* form a group of five (Yps1, Yps2, Yps3, Yps6, and Yps7) putative GPI-anchored aspartyl proteases. The yapsin family is required for the maintenance of yeast cell wall integrity and appears to be involved principally in cell wall glucan homeostasis (102). Yps1, in particular, is part of the transcriptional response to cell wall stress, through the PKC pathway, and is required during severe cell wall stress in *S. cerevisiae* (102). Similarly, *Ca*Sap9 and *Ca*Sap10 are involved in maintaining a robust cell wall in *C. albicans*. Albrecht et al. demonstrated that Sap9 and Sap10 are required for cell surface integrity and that they cleave their target proteins at similar or identical processing sites. However, each protease seems to have distinct target proteins, since the single mutants showed phenotypes that were only partially compensated for by overexpression of its paralogous Sap isoenzyme (3). In addition, they showed that *sap9*Δ and *sap10*Δ mutants had opposite adhesion properties. Mutants lacking Sap9 showed more adherence to buccal epithelial cells, the loss of Sap10 caused reduced adherence, and mutants lacking both genes behaved like the *sap10*Δ mutant. Thus, the authors speculated that Sap9 and Sap10 might have defined functions during infection similar to those of surface-associated proteases of malaria and *Toxoplasma* parasites. *SAP9* is the most highly and constitutively expressed *SAP* gene in numerous in vitro and in vivo models, such as reconstituted human epithelial models (oral and vaginal) (149), a murine parenchymal organ invasion model (50), and an immunodeficiency mouse model for oropharyngeal infections (179), suggesting a role during growth in contact with host cells. In addition, *SAP9* gene expression increases upon treatment of *C. albicans* with fluconazole and caspofungin (35). In a recent study, Sap9 rather than Sap10 was found to be necessary for efficient recognition and killing of *C. albicans* by human polymorphonuclear leukocytes (PMNs) (75). Deletion of *SAP9* decreased targeted motility of PMNs towards *C. albicans*. It has been proposed that loss of a substrate of Sap9, which is released by *C. albicans*, rather than Sap9 itself, may lead to PMN activation (75). In conclusion, fungal yapsins appear to constitute a special class of aspartic endopeptidases characterized by their restricted substrate specificity, their modification by a GPI moiety, and their localization at the cell surface (60, 61). Most importantly, they are found only in fungi and are emerging as key players in cell wall assembly and/or remodeling. Since a direct action on cell wall polysaccharides is very unlikely, two activities can be suggested. One is activation of cell envelope enzymes directly involved in β-glucan synthesis, cross-linking, and remodeling by proteolysis, like Kex2 activates Exg1 in *S. cerevisiae* (13). The alternative is by catalyzing shedding of cell surface mannoproteins. On the other hand, the recent report of Sap9 action during neutrophil interaction opens a new field of investigation strictly related to the virulence traits of *C. albicans*.

Cell Wall-Localized Sods and Plb

In *C. albicans*, some members of the Sod family (Sod4 to -6) and the Plb family (Plb3 to -5) are GPI anchored. Sod and Plb proteins are catalytic enzymes either surface localized or secreted in *C. albicans*. Their location at the cell surface has been related to fungal virulence traits. Sods have a role in combating the free radicals generated by the host's immune system in response to invasive microorganisms. Mutations in *SOD4* and *SOD5* led to hypersensitivity to oxidative stresses (53, 57, 129). Plb have also been proposed as virulence factors contributing to the destruction of host membrane. Supporting this, a strain with a *PLB5* deletion produced reduced fungal burdens when tested in the mouse systemic infection model (201). This suggests a direct or indirect link between Plb activity at the cell surface and virulence, although the mechanism has not been deciphered.

Approaches To Study Fungal Glycoproteins

In order to study fungal cell wall remodeling, we first need to understand the roles of the different CWPs and we need to monitor their expression, localization, and turnover. Analyses of fungal cell wall protein function and localization are hampered because of technical issues related to their posttranslational processing. As a cell wall protein gene is translated, it is targeted to the secretory pathway passing through the ER and Golgi complex where the protein is glycosylated by the addition of predominantly O-glycan but also N-glycan. Cell wall proteins, therefore, are not resolved well by one- or two-dimensional acrylamide gel electrophoresis and appear as a high-molecular-weight smear due to heterogeneity in the addition of glycans. Removal of the glycans by endo-β-*N*-acetylglucosaminidase H or peptide *N*-glycosidase F treatment reduces the smear to a lower-molecular-weight band. Localization of glycoproteins by introducing an epitope tag or fusing with a fluorescent protein is also not trivial. Processing of protein to its mature form involves truncation at both the N and C termini, so any tagging must take place internally. In addition, a number of glycoproteins have potential Kex2 cleavage sites, suggestive of further processing by proteolytic cleavage (10). Despite these difficulties, several approaches have been adopted that circumvent the problems associated with posttranslational modifications of cell wall proteins. Successful strategies for tagging CWPs include introducing GFP or an epitope tag downstream of the signal peptide or upstream of the GPI anchor attachment site. Using a simple PCR strategy with primers that contain the sequence encoding the hemagglutinin (HA) epitope of influenza virus, Li et al. introduced an HA tag in the Eap1 coding sequence, allowing the localization after digestion of the cell wall of the tagged strain (116). Other laboratories have used the V5 tag and localized Pir1 or Dfg5 using this technique (130, 194). A large number of proteins have been localized using GFP fusions, for example, Pga59, Pga62, Sod5, Sap9, Sap10, Hwp1, and Ecm331, either directly by fluorescence microscopy or by immunoelectron microscopy and gold particles (3, 53, 126, 127, 135). Examples

of antibodies raised against cell surface proteins are scarce; Rbt5 was one of the few proteins localized through this type of strategy (213).

The poor resolution of fungal glycoproteins through SDS-polyacrylamide gel electrophoresis has prompted alternative nongel technologies to study the complement of proteins in the cell wall. One such method is the use of liquid chromatography and tandem mass spectrometry (40, 219). Purified cell walls are subjected to trypsin digestion and the peptides released separated by mass and then identified by mass spectrometry. This method has identified around 30 *C. albicans* cell wall proteins, depending upon the growth conditions and morphology (30, 40, 192).

Heterologous expression of the complete glycoprotein or partial fragments, such as the N-terminal functional domain, has provided some insights into the function of several *Candida* cell wall proteins. One popular approach is to express *Candida* proteins on the surface of the rather poorly adherent *S. cerevisiae* and then assay for increased adhesion to a variety of substrates, including innate surfaces, host proteins, primary epithelial cells, epithelial and endothelial cell lines, and glycan arrays. This approach led to the identification of the Eap1 (114) and Ala1/Als5 (68) adhesins of *C. albicans*. Expression of members of the Als family in *S. cerevisiae* and the swapping of specific domains demonstrated that different family members have different specificities of binding to a range of host proteins (191). The N-terminal immunoglobulin G-like domain of Als5 as well as the threonine-rich tandem repeats confer fibronectin-binding and cell-to-cell adhesion properties on Als5 (175) (Fig. 5). The glycan binding specificity of three members of the *C. glabrata* Epa family (see chapter 16) has been identified by expressing their N-terminal domains as fusion proteins on the surface of *S. cerevisiae* and measuring the affinity of the yeast strains to a vast range of glycans immobilized on a microarray (225). Epa1, Epa6, and Epa7 were found to bind to glycans with a terminal galactose. Epa6 had a broader specificity than the closely related Epa7, and the difference was mapped to a small region of 5 aa (225).

Overview of Cell Surface Protein Functions

Cell surface proteins and virulence are logically linked when describing the biology of a pathogen for the simple reason of spatial location. A large number of cell surface proteins are involved in virulence; in *C. albicans* the expression of covalently linked CWPs is tightly controlled, and they have been proposed to play a crucial role in fitness as well as virulence (95).

C. albicans has evolved numerous mechanisms that enable it to survive in the host, to disseminate, and to invade host tissues and organs, leading to a life-threatening infection if not diagnosed and treated quickly. Interestingly, CWPs seem to be implicated in many, if not all, of these different "virulence traits." Some of these virulence traits, such as adhesion and biofilm formation, are described in detail elsewhere in this book and covered in a number of review articles and so are mentioned only briefly here (31, 95, 144). CWPs have a strong role in biofilm formation; particularly involved are Als1, Als3, Hwp1, Csa1, Pga10, Rbt5, and Eap1 (14, 66, 96, 154, 155, 161). CWPs are also implicated in a number of other attributes, such as cell wall hydrophobicity and adhesion (Eap1) (114), *Candida-Candida* and *Candida*-host adhesion (58, 97, 114, 122, 195), iron acquisition (Als3 and the Rbt proteins) (4, 213, 214), morphogenesis, and resistance to stress (43, 131, 135, 177). A number of different CWPs are involved in cell wall remodeling (Bgl2, Pga31, Gas family, Crh family, Ecm33.3, and Kre1). In addition, others play important roles in detoxification (Sod proteins) (53, 57, 129) and the direct attack of host components (Saps and Plb) (3, 75, 201).

In conclusion, cell surface glycoproteins are obviously of central importance for the growth of *C. albicans* within its host, but globally their functions are still very poorly understood. Indeed, in 2007, 65% of the GPI-anchored proteins had no known function; 56% remain uncharacterized even though mutant construction has been very active, with 64 mutants available now, versus 28 in 2007 (M. L. Richard, unpublished data). Many mutants lacking a single CWP display no phenotype under the conditions tested, probably because the conditions used are not those where the protein is expressed and/or active. Another reason may be functional redundancy between different family members or compensatory effects due to loss of a specific protein. The compensatory effect may be generated by proteins with no similarity to the targeted protein. Adhesins, for example, have some specificity for different substrates, but it is very likely that they compensate for each other at different levels.

The difficulty now is not in the development of new tests to characterize these numerous mutants, because very different models have been set up for a wide range of infection-related phenotypes like biofilm formation or in vivo models. The difficulty is in choosing the right test among the constantly increasing assays produced. One way to address this would be to know the conditions of expression of these proteins, considering that under these conditions the protein is more likely to have a "visible" or measurable function. So far, no systematic analysis of CWP expression has been performed. However, global analyses of the transcriptome under a large variety of environmental conditions, including those that mimic growth in vivo, challenge with immune cells, and from in vivo models, have highlighted the increased expression of a number of *CWP* genes (63, 66, 79, 120).

COORDINATED REGULATION OF CELL WALL BIOSYNTHESIS

There are now several lines of evidence that show that the fungal cell wall is a dynamic organelle that responds to internal and external stimuli. The ability of the fungus to modify its cell wall architecture is an important survival mechanism that ensures that cellular integrity is maintained, combating sometimes potentially lethal assaults that damage the cell wall. For example, in response to sub-MIC concentrations of the glucan synthase inhibitors, the echinocandin drugs, *C. albicans* can activate chitin biosynthesis to combat the reduction in β(1,3)-glucan levels and maintain a robust cell wall (211). This salvage mechanism involves the PKC integrity pathway, the HOG pathway, and the calcium/calcineurin signaling pathway (Fig. 1). Elevated chitin levels are due mainly to *Ca*Chs3. Similarly increasing cell wall chitin through simultaneous activation of the PKC and calcium pathways (by growing cells in the presence of calcium and CFW) reduces echinocandin susceptibility (211).

In *S. cerevisiae*, cell wall damage is detected by membrane-bound sensors, such as the nano-spring-like Wsc1 that lies upstream of the PKC integrity pathway (45). A number of sensors that transduce perturbations in the cell wall have been characterized in *S. cerevisiae* (112). Transcript profiling has provided information to identify the sensor that is responding to a particular cell wall perturba-

tion, which ultimately activates a signature of cell wall salvage genes, such as *Sc*GFA1 and *Sc*CRH1. The sensors *Sc*Wsc1 and *Sc*Mid2 are responsible for sensing cell wall damage caused by Congo red and echinocandins, whereas *Sc*Msb2 and *Sc*Hkr1, two signaling-type mucins, detect damage caused by zymolyase (Fig. 1) (8, 64, 199). Furthermore, *S. cerevisiae* Mpk1/Slt2 has been to shown to activate expression of the *FKS2* glucan synthase subunit in the absence of kinase activity by forming a complex with the Swi4 subunit of SBF (92).

In *C. albicans*, the Cek1 signaling pathway also plays an important role in cell wall damage responses as well as chlamydospore formation (49), sensitivity to phagocyte-mediated killing (7), mating (218), and quorum sensing (182). Interestingly, the Cek1 pathway also promotes immune evasion, as the *cek1*Δ mutant has enhanced glucan exposure and triggers elevated dectin-1-mediated immune responses (62). In *C. albicans*, the Sho1 adaptor protein lies upstream of Cek1 (5). The *C. albicans* Msb2 orthologue is also required for activation of the Cek1 mitogen-activated protein (MAP) kinase pathway in response to caspofungin, Congo red, Zymolyase, and tunicamycin (183). In addition, Cdc42 plays a role in mediating the signal that results in Cek1 phosphorylation, and Msb2, as well as Sho1 and Hst7, lies upstream of Cdc42 (Fig. 1) (183). The *sho1*Δ, *cek1*Δ, and *chk1*Δ null mutants share common phenotypes, including Congo red hypersensitivity. Chk1 is a histidine kinase involved in the regulation of *C. albicans* mannan biosynthesis (101). In fungi, these form part of a three-component phosphotransfer relay system composed of a membrane-bound histidine kinase, a histidine intermediate protein, and a response regulator. To determine whether Chk1, like Sho1, lies upstream of Cek1, the phenotype of a double *sho1*Δ *chk1*Δ mutant was compared to those of the single *sho1*Δ, *cek1*Δ, and *chk1*Δ mutants (113). This demonstrated that Chk1 and Cek1 have a functional relationship in mannan biosynthesis that requires Sho1 only in part. However, in terms of the response to Congo red, Chk1 belonged to a pathway similar to but distinct from that of Sho1 and Cek1 (113). These studies highlight the complex interaction networks that regulate cell wall biosynthesis.

Cell Wall Remodeling and Its Impact on Pathogenesis

A number of external stimuli trigger coordinated changes in the architecture of the cell wall. Perhaps the best studied is the induction of morphogenesis. As *C. albicans* morphs between yeast, pseudohyphae, and hyphae, a number of changes occur at the cell surface; the hyphal cell wall is very different from the yeast cell wall. A variety of signals, such as increased temperature (37°C) and neutral pH, as well as addition of serum or *N*-acetylglucosamine, can trigger hyphal formation in vitro. Common to all these stimuli is induction of chitin synthesis, with hyphal cell walls having three- to fivefold-higher chitin levels (33, 146, 197). Expression analyses have revealed a number of proteins that are selectively associated with hyphal growth, such as Hwp1 (196), Hyr1 (12), Als3/Als8 (78), Rbt1 (17), Csa1/Wap1 (17), and Ihd1/Pga36 (151), whereas Ywp1 appears to be associated with growth in the yeast form (71). These changes to the cell surface between yeast cells and hyphae potentially alter the antigenicity of *C. albicans* as well as altering the fungal components exposed to a host's immune cell receptors and therefore induce different immune responses (see chapter 11).

Other cell wall proteins are regulated by pH (Phr1 and Phr2) (52, 143, 186). Phr1 and Phr2 have a reciprocal expression pattern, with Phr1 expressed at neutral pH and Phr2 at more acidic pH. They are both members of the Gas-like family in *C. albicans*, which also includes Pga4 (47) and Pga5, which are not regulated by pH. *PGA4* expression was induced in an early stage of epithelium infection, while neither *PGA5* nor *PHR3* was induced (47). As mentioned above, the Gas family of *S. cerevisiae* consists of important transglycosidases/glycosyltransferases that modify β(1,3)-glucan (28, 139, 172, 173). At least two members are developmentally regulated: Gas2 and Gas4 are required for the correct assembly of the *S. cerevisiae* spore wall (172). Members of the Gas family play a central role in the remodeling of the cell wall in response to cell wall defects. The *gas1*Δ mutant in *S. cerevisiae* has elevated chitin levels, and a higher proportion of cell wall proteins are linked to chitin rather than β(1,3)-glucan in this mutant (168). As a consequence, Chs3 activity is essential for viability of the *gas1*Δ mutant (27, 168, 207). The importance of glucan synthesis, the glucan remodeling activity of the Gas proteins, and chitin synthesis to cell viability is reflected in the synthetic lethality observed between these crucial cell wall manufacturing processes (110, 111). Although fungi can adapt to grow in the absence of one of these activities by upregulating the other two processes, viability is lost when two or more of these activities are impaired.

Expression analyses with DNA microarrays at the mRNA level or proteomics to look at posttranslational regulation have highlighted a number of changes in the cell wall proteome in response to changing growth conditions and to cell wall perturbations, for example, treatment with the echinocandin drugs, in vivo growth, biofilm formation, and signaling pathway agonists and antagonists. Altered expression levels not only affect the cell wall proteome but also have a knock-on effect in terms of remodeling the cell wall polysaccharides and could potentially lead to significant changes in the cell wall. This may change the physical characteristics of the wall, its rigidity, hydrophobic properties, charge, and adhesiveness and the exposure of antigenic proteins, as well as ligands of innate immunity receptors. All of these are likely to impinge on the interactions of *Candida* species with their host in commensalism, colonization, and disease processes as well as interactions with other microbes present in the same niches.

Our understanding of the way the cell wall is altered when the fungus is growing in the host is rudimentary. However, there is evidence that the in vivo cell wall may be significantly different from the cell wall of in vitro-grown cells, which has been well characterized and the focus of most studies. As *C. albicans* disseminates throughout the body it is exposed to a variety of environmental conditions: variable pH and oxygen availability and different sources of nutrients. In vitro growth conditions that mimic in vivo conditions, for example, the use of hypoxia and artificial vaginal fluid, have influenced the cell wall proteome (192). Transcript profiling of *C. albicans* cells from in vivo models (see chapter 18) has identified a number of cell wall-associated genes whose expression is altered compared to those in in vitro-grown cells. As well as being exposd to different stimuli that are independently known to alter expression of cell wall-related genes, the invading fungus will also be under attack by the host's enzymes and immune cells. There is evidence that β(1,3)-glucan is released from fungal cells during infection. In vitro-grown yeast and hyphal cells have been shown to release CWP epitopes into the medium (204), and

it is highly likely that the same process takes place in vivo. Wheeler et al. (215) have also demonstrated that β(1,3)-glucan becomes more surface exposed, predominantly on hyphal cells at later stages of infection. As β(1,3)-glucan acts as the main scaffold of the cell wall anchoring the cell wall proteins via the β(1,6)-glucan linker, this is likely to be accompanied by alterations in the outer mannoprotein layer. In addition, treating cells with sublethal doses of the echinocandin drugs can result in significant remodeling of the cell wall. This can take the form of elevated chitin levels but also can result in enhanced exposure of β(1,3)-glucans on the fungal surface that would result in increased recognition by the PRR dectin-1 (215). The ability to detect circulating β(1,3)-glucan is currently the basis of a commercially available diagnosis kit for invasive fungal infections, and although it is highly sensitive, there are some problems with false positives (158, 189). Sera from patients suffering from different forms of candidiasis contain antibodies against a number of *C. albicans* proteins: both intracellular proteins, such as enolase and Hsp90, and surface proteins, like Bgl2 (118, 164–166). Therefore, there is great potential to develop novel diagnostics as well as vaccines based on cell surface antigens. Much effort has already focused on identifying potential candidates for development of vaccines (29, 37). The N-terminal regions of Als1 and Als3 (193), synthetic glycopeptides that are based on epitopes of cell wall-associated proteins conjugated to mannose trisaccharides (216), and β-glucan conjugates have proven efficacious in mouse models of systemic mycoses (18, 163).

CONCLUSIONS

The cell wall plays a vital role in maintaining cellular integrity by protecting cells from external traumas and by adapting its architecture in response to a variety of stimuli. In fungi that undergo morphogenesis, like *C. albicans*, there is coordinated regulation of cell wall synthesis to enable the cell to change shape and switch from isotropic to polarized growth. Although we know the fundamental principles underlying cell wall biogenesis and its regulation, we still have much to learn about how the different components of the cell wall are assembled into the mature wall and how this is coordinated. In particular, the processes and proteins involved in tethering GPI-anchored proteins to the wall, cell wall protein turnover, and the interactions between proteins in the wall remain largely uncharacterized. The cell wall harbors important virulence attributes and immune reactive molecules that contribute to fungal pathogenesis, forms an intimate interface with the host and the host's microbiota in commensalism and disease, and remains a highly attractive target for novel therapies and diagnostics. In addition, modulation of the cell wall can affect antifungal susceptibility and immune evasion. For all these reasons, the fungal wall remains a stimulating subject of research.

REFERENCES

1. **Aimanianda, V., C. Clavaud, C. Simenel, T. Fontaine, M. Delepierre, and J. P. Latge.** 2009. Cell wall beta-(1,6)-glucan of *Saccharomyces cerevisiae*: structural characterization and in situ synthesis. *J. Biol. Chem.* **284:** 13401–13412.
2. **Alberti-Segui, C., A. J. Morales, H. Xing, M. M. Kessler, D. A. Willins, K. G. Weinstock, G. Cottarel, K. Fechtel, and B. Rogers.** 2004. Identification of potential cell-surface proteins in *Candida albicans* and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence. *Yeast* **21:**285–302.
3. **Albrecht, A., A. Felk, I. Pichova, J. R. Naglik, M. Schaller, P. De Groot, D. MacCallum, F. C. Odds, W. Schafer, F. Klis, M. Monod, and B. Hube.** 2006. Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. *J. Biol. Chem.* **281:** 688–694.
4. **Almeida, R. S., S. Brunke, A. Albrecht, S. Thewes, M. Laue, J. E. Edwards, S. G. Filler, and B. Hube.** 2008. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog.* **4:**e1000217.
5. **Alonso-Monge, R., E. Roman, D. M. Arana, J. Pla, and C. Nombela.** 2009. Fungi sensing environmental stress. *Clin. Microbiol. Infect.* **15**(Suppl. 1)**:**17–19.
6. **Angiolella, L., A. Vitali, A. Stringaro, G. Mignogna, B. Maras, M. Bonito, M. Colone, A. T. Palamara, and A. Cassone**. 2009. Localisation of Bgl2p upon antifungal drug treatment in *Candida albicans*. *Int. J. Antimicrob. Agents* **33:**143–148.
7. **Arana, D. M., R. Alonso-Monge, C. Du, R. Calderone, and J. Pla.** 2007. Differential susceptibility of mitogen-activated protein kinase pathway mutants to oxidative-mediated killing by phagocytes in the fungal pathogen *Candida albicans*. *Cell. Microbiol.* **9:**1647–1659.
8. **Arroyo, J., C. Bermejo, R. Garcia, and J. M. Rodriguez-Pena.** 2009. Genomics in the detection of damage in microbial systems: cell wall stress in yeast. *Clin. Microbiol. Infect.* **15**(Suppl. 1)**:**44–46.
9. **Arroyo, J., J. Sarfati, M. T. Baixench, E. Ragni, M. Guillen, J. M. Rodriguez-Pena, L. Popolo, and J. P. Latge.** 2007. The GPI-anchored Gas and Crh families are fungal antigens. *Yeast* **24:**289–296.
10. **Bader, O., Y. Krauke, and B. Hube**. 2008. Processing of predicted substrates of fungal Kex2 proteinases from *Candida albicans*, *C. glabrata*, *Saccharomyces cerevisiae* and *Pichia pastoris*. *BMC Microbiol.* **8:**116.
11. **Bailey, D.** 1997. Cloning and characterisation of the *HYR1* and *UBI4* genes from *Candida albicans*. Ph.D. thesis. University of Aberdeen, Aberdeen, United Kingdom.
12. **Bailey, D. A., P. J. F. Feldmann, M. Bovey, N. A. R. Gow, and A. J. P. Brown.** 1996. The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J. Bacteriol.* **178:**5353–5360.
13. **Basco, R. D., R. Cueva, E. Andaluz, and G. Larriba.** 1996. *In vivo* processing of the precursor of the major exoglucanase by KEX2 endoprotease in the *Saccharomyces cerevisiae* secretory pathway. *Biochim. Biophys. Acta* **1310:** 110–118.
14. **Bastidas, R. J., J. Heitman, and M. E. Cardenas.** 2009. The protein kinase Tor1 regulates adhesin gene expression in *Candida albicans*. *PLoS Pathog.* **5:**e1000294.
15. **Bates, S., J. M. de la Rosa, D. M. MacCallum, A. J. Brown, N. A. Gow, and F. C. Odds.** 2007. *Candida albicans* Iff11, a secreted protein required for cell wall structure and virulence. *Infect. Immun.* **75:**2922–2928.
16. **Brand, A., S. Shanks, V. M. Duncan, M. Yang, K. Mackenzie, and N. A. Gow.** 2007. Hyphal orientation of *Candida albicans* is regulated by a calcium-dependent mechanism. *Curr. Biol.* **17:**347–352.
17. **Braun, B. R., W. S. Head, M. X. Wang, and A. D. Johnson.** 2000. Identification and characterization of *TUP1*-regulated genes in *Candida albicans*. *Genetics* **156:**31–44.
18. **Bromuro, C., M. Romano, P. Chiani, F. Berti, M. Tontini, D. Proietti, E. Mori, A. Torosantucci, P. Costan-**

tino, R. Rappuoli, and A. Cassone. 2010. Beta-glucan-CRM197 conjugates as candidates antifungal vaccines. *Vaccine* **28:**2615–2623.
19. **Brown, G. D., P. R. Taylor, D. M. Reid, J. A. Willment, D. L. Williams, L. Martinez-Pomares, S. Y. Wong, and S. Gordon.** 2002. Dectin-1 is a major beta-glucan receptor on macrophages. *J. Exp. Med.* **196:**407–412.
20. **Brown, J. A., and B. J. Catley.** 1992. Monitoring polysaccharide synthesis in *Candida albicans*. *Carbohydr. Res.* **227:**195–202.
21. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J. L. Reedy, I. Agrafioti, M. B. Arnaud, S. Bates, A. J. Brown, S. Brunke, M. C. Costanzo, D. A. Fitzpatrick, P. W. De Groot, D. Harris, L. L. Hoyer, B. Hube, F. M. Klis, C. Kodira, N. Lennard, M. E. Logue, R. Martin, A. M. Neiman, E. Nikolaou, M. A. Quail, J. Quinn, M. C. Santos, F. F. Schmitzberger, G. Sherlock, P. Shah, K. A. Silverstein, M. S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M. P. Stumpf, P. E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N. A. Gow, M. C. Lorenz, B. W. Birren, M. Kellis, and C. A. Cuomo.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459:**657–662.
22. **Cabezon, V., A. Llama-Palacios, C. Nombela, L. Monteoliva, and C. Gil.** 2009. Analysis of *Candida albicans* plasma membrane proteome. *Proteomics* **9:**4770–4786.
23. **Cabib, E.** 2009. Two novel techniques for determination of polysaccharide cross-links show that Crh1p and Crh2p attach chitin to both β(1-6)- and β(1-3)glucan in the *Saccharomyces cerevisiae* cell wall. *Eukaryot. Cell* **8:** 1626–1636.
24. **Cabib, E., N. Blanco, C. Grau, J. M. Rodriguez-Pena, and J. Arroyo.** 2007. Crh1p and Crh2p are required for the cross-linking of chitin to beta(1-6)glucan in the *Saccharomyces cerevisiae* cell wall. *Mol. Microbiol.* **63:**921–935.
25. **Cabib, E., V. Farkas, O. Kosik, N. Blanco, J. Arroyo, and P. McPhie.** 2008. Assembly of the yeast cell wall. Crh1p and Crh2p act as transglycosylases *in vivo* and *in vitro*. *J. Biol. Chem.* **283:**29859–29872.
26. **Cantarel, B. L., P. M. Coutinho, C. Rancurel, T. Bernard, V. Lombard, and B. Henrissat.** 2009. The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* **37:** D233–D238.
27. **Carotti, C., L. Ferrario, C. Roncero, M.-H. Valdivieso, A. Duran, and L. Popolo.** 2002. Maintenance of cell integrity in the *gas1* mutant of *Saccharomyces cerevisiae* requires the Chs3p-targeting and activation pathway and involves an unusual Chs3p localization. *Yeast* **19:**1113–1124.
28. **Carotti, C., E. Ragni, O. Palomares, T. Fontaine, G. Tedeschi, R. Rodriguez, J. P. Latge, M. Vai, and L. Popolo.** 2004. Characterization of recombinant forms of the yeast Gas1 protein and identification of residues essential for glucanosyltransferase activity and folding. *Eur. J. Biochem.* **271:**3635–3645.
29. **Cassone, A.** 2008. Fungal vaccines: real progress from real challenges. *Lancet Infect. Dis.* **8:**114–124.
30. **Castillo, L., E. Calvo, A. I. Martinez, J. Ruiz-Herrera, E. Valentin, J. A. Lopez, and R. Sentandreu.** 2008. A study of the *Candida albicans* cell wall proteome. *Proteomics* **8:**3871–3881.
31. **Chaffin, W. L.** 2008. *Candida albicans* cell wall proteins. *Microbiol. Mol. Biol. Rev.* **72:**495–544.
32. **Chaffin, W. L., J. L. Lopez-Ribot, M. Casanova, D. Gozalbo, and J. P. Martinez.** 1998. Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. *Microbiol. Mol. Biol. Rev.* **62:**130–180.
33. **Chattaway, F. W., M. R. Holmes, and A. J. E. Barlow.** 1968. Cell wall composition of the mycelial and blastospore forms of *Candida albicans*. *J. Gen. Microbiol.* **51:**367–376.
34. **Cleves, A. E., D. N. Cooper, S. H. Barondes, and R. B. Kelly.** 1996. A new pathway for protein export in *Saccharomyces cerevisiae*. *J. Cell Biol.* **133:**1017–1026.
35. **Copping, V. M., C. J. Barelle, B. Hube, N. A. Gow, A. J. Brown, and F. C. Odds.** 2005. Exposure of *Candida albicans* to antifungal agents affects expression of *SAP2* and *SAP9* secreted proteinase genes. *J. Antimicrob. Chemother.* **55:** 645–654.
36. **Cote, P., and M. Whiteway.** 2008. The role of *Candida albicans FAR1* in regulation of pheromone-mediated mating, gene expression and cell cycle arrest. *Mol. Microbiol.* **68:**392–404.
37. **Cutler, J. E., G. S. Deepe, Jr., and B. S. Klein.** 2007. Advances in combating fungal diseases: vaccines on the threshold. *Nat. Rev. Microbiol.* **5:**13–28.
38. **De Bernardis, F., F. A. Muhlschlegel, A. Cassone, and W. A. Fonzi.** 1998. The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect. Immun.* **66:**3317–3325.
39. **De Groot, P. W., B. W. Brandt, and F. M. Klis.** 2007. Cell wall biology of *Candida*, p. 291–323. *In* C. d'Enfert and B. Hube (ed.), Candida: *Comparative and Functional Genomics*. Caister Academic Press, Norfolk, United Kingdom.
40. **De Groot, P. W., A. D. de Boer, J. Cunningham, H. L. Dekker, L. de Jong, K. J. Hellingwerf, C. de Koster, and F. M. Klis.** 2004. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryot. Cell* **3:**955–965.
41. **De Groot, P. W., K. J. Hellingwerf, and F. M. Klis.** 2003. Genome-wide identification of fungal GPI proteins. *Yeast* **20:**781–796.
42. **De Groot, P. W., E. A. Kraneveld, Q. Y. Yin, H. L. Dekker, U. Gross, W. Crielaard, C. G. De Koster, O. Bader, F. M. Klis, and M. Weig.** 2008. The cell wall of the human pathogen *Candida glabrata*: differential incorporation of novel adhesin-like wall proteins. *Eukaryot. Cell* **7:**1951–1964.
43. **De Groot, P. W., A. F. Ram, and F. M. Klis.** 2005. Features and functions of covalently linked proteins in fungal cell walls. *Fungal Genet. Biol.* **42:**657–675.
44. **Douglas, C. M., J. A. D'Ippolito, G. J. Shei, M. Meinz, J. Onishi, J. A. Marrinan, W. Li, G. K. Abruzzo, A. Flattery, K. Bartizal, A. Mitchell, and M. B. Kurtz.** 1997. Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1,3-β-D-glucan synthase inhibitors. *Antimicrob. Agents Chemother.* **41:**2471–2479.
45. **Dupres, V., D. Alsteens, S. Wilk, B. Hansen, J. J. Heinisch, and Y. F. Dufrene.** 2009. The yeast Wsc1 cell surface sensor behaves like a nanospring in vivo. *Nat. Chem. Biol.* **5:**857–862.
46. **Ecker, M., R. Deutzmann, L. Lehle, V. Mrsa, and W. Tanner.** 2006. Pir proteins of *Saccharomyces cerevisiae* are attached to beta-1,3-glucan by a new protein-carbohydrate linkage. *J. Biol. Chem.* **281:**11523–11529.
47. **Eckert, S. E., W. J. Heinz, K. Zakikhany, S. Thewes, K. Haynes, B. Hube, and F. A. Muhlschlegel.** 2006. *PGA4*, a GAS homologue from *Candida albicans*, is up-regulated early in infection processes. *Fungal Genet. Biol.* **44:**368–377.
48. **Eisenhaber, B., G. Schneider, M. Wildpaner, and F. Eisenhaber.** 2004. A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *J. Mol. Biol.* **337:** 243–253.

49. **Eisman, B., R. Alonso-Monge, E. Roman, D. Arana, C. Nombela, and J. Pla.** 2006. The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. *Eukaryot. Cell* **5:**347–358.
50. **Felk, A., M. Kretschmar, A. Albrecht, M. Schaller, S. Beinhauer, T. Nichterlein, D. Sanglard, H. C. Korting, W. Schafer, and B. Hube.** 2002. *Candida albicans* hyphal formation and the expression of the Efg1-regulated proteinases Sap4 to Sap6 are required for the invasion of parenchymal organs. *Infect. Immun.* **70:**3689–3700.
51. **Ferguson, M. A., S. W. Homans, R. A. Dwek, and T. W. Rademacher.** 1988. Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. *Science* **239:**753–759.
52. **Fonzi, W. A.** 1999. *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of β-1,3- and β-1,6-glucans. *J. Bacteriol.* **181:** 7070–7079.
53. **Fradin, C., P. De Groot, D. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube.** 2005. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol. Microbiol.* **56:**397–415.
54. **Frank, A. T., C. B. Ramsook, H. N. Otoo, C. Tan, G. Soybelman, J. M. Rauceo, N. K. Gaur, S. A. Klotz, and P. N. Lipke.** 2010. Structure and function of glycosylated tandem repeats from *Candida albicans* Als adhesins. *Eukaryot. Cell* **9:**405–414.
55. **Frieman, M. B., and B. P. Cormack.** 2003. The omega-site sequence of glycosylphosphatidylinositol-anchored proteins in *Saccharomyces cerevisiae* can determine distribution between the membrane and the cell wall. *Mol. Microbiol.* **50:**883–896.
56. **Frieman, M. B., and B. P. Cormack.** 2004. Multiple sequence signals determine the distribution of glycosylphosphatidylinositol proteins between the plasma membrane and cell wall in *Saccharomyces cerevisiae*. *Microbiology* **150:**3105–3114.
57. **Frohner, I. E., C. Bourgeois, K. Yatsyk, O. Majer, and K. Kuchler.** 2009. *C. albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. *Mol. Microbiol.* **71:**240–252.
58. **Fu, Y., A. S. Ibrahim, D. C. Sheppard, Y. C. Chen, S. W. French, J. E. Cutler, S. G. Filler, and J. E. Edwards.** 2002. *Candida albicans* Als1p: an adhesin that is a downstream effector of the *EFG1* filamentation pathway. *Mol. Microbiol.* **44:**61–72.
59. **Fu, Y., G. Luo, B. J. Spellberg, J. E. Edwards, Jr., and A. S. Ibrahim.** 2008. Gene overexpression/suppression analysis of candidate virulence factors of *Candida albicans*. *Eukaryot. Cell* **7:**483–492.
60. **Gagnon-Arsenault, I., L. Parise, J. Tremblay, and Y. Bourbonnais.** 2008. Activation mechanism, functional role and shedding of glycosylphosphatidylinositol-anchored Yps1p at the *Saccharomyces cerevisiae* cell surface. *Mol. Microbiol.* **69:**982–993.
61. **Gagnon-Arsenault, I., J. Tremblay, and Y. Bourbonnais.** 2006. Fungal yapsins and cell wall: a unique family of aspartic peptidases for a distinctive cellular function. *FEMS Yeast Res.* **6:**966–978.
62. **Galan-Diez, M., D. M. Arana, D. Serrano-Gomez, L. Kremer, J. M. Casasnovas, M. Ortega, A. Cuesta-Dominguez, A. L. Corbi, J. Pla, and E. Fernandez-Ruiz.** 2010. *Candida albicans* β-glucan exposure is controlled by the fungal *CEK1*-mediated mitogen-activated protein kinase pathway that modulates immune responses triggered through dectin-1. *Infect. Immun.* **78:**1426–1436.
63. **Garcera, A., L. Castillo, A. I. Martinez, M. V. Elorza, E. Valentin, and R. Sentandreu.** 2005. Anchorage of *Candida albicans* Ssr1 to the cell wall, and transcript profiling of the null mutant. *Res. Microbiol.* **156:**911–920.
64. **Garcia, R., J. M. Rodriguez-Pena, C. Bermejo, C. Nombela, and J. Arroyo.** 2009. The high osmotic response and cell wall integrity pathways cooperate to regulate transcriptional responses to zymolyase-induced cell wall stress in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **284:**10901–10911.
65. Reference deleted.
66. **Garcia-Sanchez, S., S. Aubert, I. Iraqui, G. Janbon, J. M. Ghigo, and C. d'Enfert.** 2004. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot. Cell* **3:**536–545.
67. **Gastebois, A., I. Mouyna, C. Simenel, C. Clavaud, B. Coddeville, M. Delepierre, J. P. Latge, and T. Fontaine.** 2010. Characterization of a new beta (1-3)-glucan branching activity of *Aspergillus fumigatus*. *J. Biol. Chem.* **285:**2386–2396.
68. **Gaur, N. K., S. A. Klotz, and R. L. Henderson.** 1999. Overexpression of the *Candida albicans ALA1* gene in *Saccharomyces cerevisiae* results in aggregation following attachment of yeast cells to extracellular matrix proteins, adherence properties similar to those of *Candida albicans*. *Infect. Immun.* **67:**6040–6047.
69. **Gil-Navarro, I., M. L. Gil, M. Casanova, J. E. O'Connor, J. P. Martinez, and D. Gozalbo.** 1997. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is a surface antigen. *J. Bacteriol.* **179:** 4992–4999.
70. **Gomez, M. J., A. Torosantucci, S. Arancia, B. Maras, L. Parisi, and A. Cassone.** 1996. Purification and biochemical characterization of a 65-kilodalton mannoprotein (MP65), a main target of anti-*Candida* cell-mediated immune responses in humans. *Infect. Immun.* **64:**2577–2584.
71. **Granger, B. L., M. L. Flenniken, D. A. Davis, A. P. Mitchell, and J. E. Cutler.** 2005. Yeast wall protein 1 of *Candida albicans*. *Microbiology* **151:**1631–1644.
72. **Hamada, K., H. Terashima, M. Arisawa, and K. Kitada.** 1998. Amino acid sequence requirement for efficient incorporation of glycosylphosphatidylinositol-associated proteins into the cell wall of *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273:**26946–26953.
73. **Hartland, R. P., T. Fontaine, J. P. Debeaupuis, C. Simenel, M. Delepierre, and J. P. Latge.** 1996. A novel beta-(1-3)-glucanosyltransferase from the cell wall of *Aspergillus fumigatus*. *J. Biol. Chem.* **271:**26843–26849.
74. **Hong, Z., P. Mann, K. J. Shaw, and B. J. Di Domenico.** 1994. Analysis of beta-glucans and chitin in a *Saccharomyces cerevisiae* cell wall mutant using high-performance liquid chromatography. *Yeast* **10:**1083–1092.
75. **Hornbach, A., A. Heyken, L. Schild, B. Hube, J. Loffler, and O. Kurzai.** 2009. The glycosylphosphatidylinositol-anchored protease Sap9 modulates the interaction of *Candida albicans* with human neutrophils. *Infect. Immun.* **77:**5216–5224.
76. **Hoyer, L. L.** 2001. The *ALS* gene family of *Candida albicans*. *Trends Microbiol.* **9:**176–180.
77. **Hoyer, L. L., C. B. Green, S. H. Oh, and X. Zhao.** 2008. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (*ALS*) gene family—a sticky pursuit. *Med. Mycol.* **46:**1–15.
78. **Hoyer, L. L., T. L. Payne, M. Bell, A. M. Myers, and S. Scherer.** 1998. *Candida albicans ALS3* and insights into the nature of the *ALS* gene family. *Curr. Genet.* **33:** 451–459.
79. **Hube, B.** 2004. From commensal to pathogen: stage- and tissue-specific gene expression of *Candida albicans*. *Curr. Opin. Microbiol.* **7:**336–341.

80. **Hube, B., and J. Naglik.** 2001. *Candida albicans* proteinases: resolving the mystery of a gene family. *Microbiology* **147:**1997–2005.
81. **Inoue, N., Y. Murakami, and T. Kinoshita.** 2003. Molecular genetics of paroxysmal nocturnal hemoglobinuria. *Int. J. Hematol.* **77:**107–112.
82. **Iorio, E., A. Torosantucci, C. Bromuro, P. Chiani, A. Ferretti, M. Giannini, A. Cassone, and F. Podo.** 2008. *Candida albicans* cell wall comprises a branched β-D-(1→6)-glucan with β-D-(1→3)-side chains. *Carbohydr. Res.* **343:**1050–1061.
83. **Ishihara, S., A. Hirata, S. Nogami, A. Beauvais, J. P. Latge, and Y. Ohya.** 2007. Homologous subunits of 1,3-beta-glucan synthase are important for spore wall assembly in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **6:**143–156.
84. **Jaafar, L., I. Moukadiri, and J. Zueco.** 2003. Characterization of a disulphide-bound Pir-cell wall protein (Pir-CWP) of *Yarrowia lipolytica*. *Yeast* **20:**417–426.
85. **Kalebina, T. S., D. K. Laurinavichiute, A. N. Packeiser, O. S. Morenkov, M. D. Ter Avanesyan, and I. S. Kulaev.** 2002. Correct GPI-anchor synthesis is required for the incorporation of endoglucanase/glucanosyltransferase Bgl2p into the *Saccharomyces cerevisiae* cell wall. *FEMS Microbiol. Lett.* **210:**81–85.
86. **Kapteyn, J. C., G. J. Dijkgraaf, R. C. Montijn, and F. M. Klis.** 1995. Glucosylation of cell wall proteins in regenerating spheroplasts of *Candida albicans*. *FEMS Microbiol. Lett.* **128:**271–277.
87. **Kapteyn, J. C., L. L. Hoyer, J. E. Hecht, W. H. Muller, A. Andel, A. J. Verkleij, M. Makarow, H. Van den Ende, and F. M. Klis.** 2000. The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. *Mol. Microbiol.* **35:**601–611.
88. **Kapteyn, J. C., R. C. Montijn, G. J. Dijkgraaf, and F. M. Klis.** 1994. Identification of beta-1,6-glucosylated cell wall proteins in yeast and hyphal forms of *Candida albicans*. *Eur. J. Cell Biol.* **65:**402–407.
89. **Karababa, M., E. Valentino, G. Pardini, A. T. Coste, J. Bille, and D. Sanglard.** 2006. *CRZ1*, a target of the calcineurin pathway in *Candida albicans*. *Mol. Microbiol.* **59:**1429–1451.
90. **Kempf, M., V. Apaire-Marchais, P. Saulnier, P. Licznar, C. Lefrancois, R. Robert, and J. Cottin.** 2007. Disruption of *Candida albicans IFF4* gene involves modifications of the cell electrical surface properties. *Colloids Surf. B Biointerfaces* **58:**250–255.
91. **Kempf, M., J. Cottin, P. Licznar, C. Lefrancois, R. Robert, and V. Apaire-Marchais.** 2009. Disruption of the GPI protein-encoding gene *IFF4* of *Candida albicans* results in decreased adherence and virulence. *Mycopathologia* **168:**73–77.
92. **Kim, K. Y., A. W. Truman, and D. E. Levin.** 2008. Yeast Mpk1 mitogen-activated protein kinase activates transcription through Swi4/Swi6 by a noncatalytic mechanism that requires upstream signal. *Mol. Cell. Biol.* **28:**2579–2589.
93. **Klis, F. M., P. De Groot, and K. Hellingwerf.** 2001. Molecular organisation of the cell wall of *Candida albicans*. *Med. Mycol.* **39**(Suppl. 1)**:**1–8.
94. **Klis, F. M., M. de Jong, S. Brul, and P. W. De Groot.** 2007. Extraction of cell surface-associated proteins from living yeast cells. *Yeast* **24:**253–258.
95. **Klis, F. M., G. J. Sosinska, P. W. De Groot, and S. Brul.** 2009. Covalently linked cell wall proteins of *Candida albicans* and their role in fitness and virulence. *FEMS Yeast Res.* **9:**1013–1028.
96. **Klotz, S. A., N. K. Gaur, R. De Armond, D. Sheppard, N. Khardori, J. E. Edwards, Jr., P. N. Lipke, and M. El Azizi.** 2007. *Candida albicans* Als proteins mediate aggregation with bacteria and yeasts. *Med. Mycol.* **45:**363–370.
97. **Klotz, S. A., N. K. Gaur, D. F. Lake, V. Chan, J. Rauceo, and P. N. Lipke.** 2004. Degenerate peptide recognition by *Candida albicans* adhesins Als5p and Als1p. *Infect. Immun.* **72:**2029–2034.
98. **Kollar, R., E. Petrakova, G. Ashwell, P. W. Robbins, and E. Cabib.** 1995. Architecture of the yeast cell wall. The linkage between chitin and beta(1-3)-glucan. *J. Biol. Chem.* **270:**1170–1178.
99. **Kollar, R., B. B. Reinhold, E. Petrakova, H. J. C. Yeh, G. Ashwell, J. Drgonova, J. C. Kapteyn, F. M. Klis, and E. Cabib.** 1997. Architecture of the yeast cell wall: beta(1-6)-glucan interconnects mannoprotein, beta(1-3)-glucan, and chitin. *J. Biol. Chem.* **272:** 17762–17775.
100. **Kondoh, O., Y. Tachibana, Y. Ohya, M. Arisawa, and T. Watanabe.** 1997. Cloning of the *RHO1* gene from *Candida albicans* and its regulation of beta-1,3-glucan synthesis. *J. Bacteriol.* **179:**7734–7741.
101. **Kruppa, M., T. Goins, J. E. Cutler, D. Lowman, D. Williams, N. Chauhan, V. Menon, P. Singh, D. Li, and R. Calderone.** 2003. The role of the *Candida albicans* histidine kinase (*CHK1*) gene in the regulation of cell wall mannan and glucan biosynthesis. *FEMS Yeast Res.* **3:**289–299.
102. **Krysan, D. J., E. L. Ting, C. Abeijon, L. Kroos, and R. S. Fuller.** 2005. Yapsins are a family of aspartyl proteases required for cell wall integrity in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **4:**1364–1374.
103. **Kulkarni, R. D., H. S. Kelkar, and R. A. Dean.** 2003. An eight-cysteine-containing CFEM domain unique to a group of fungal membrane proteins. *Trends Biochem. Sci.* **28:**118–121.
104. **Kulkarni, R. D., M. R. Thon, H. Pan, and R. A. Dean.** 2005. Novel G-protein-coupled receptor-like proteins in the plant pathogenic fungus *Magnaporthe grisea*. *Genome Biol.* **6:**R24.
105. **Lamarre, C., N. Deslauriers, and Y. Bourbonnais.** 2000. Expression cloning of the *Candida albicans CSA1* gene encoding a mycelial surface antigen by sorting of *Saccharomyces cerevisiae* transformants with monoclonal antibody-coated magnetic beads. *Mol. Microbiol.* **35:**444–453.
106. **Lee, S. A., S. Wormsley, S. Kamoun, A. F. Lee, K. Joiner, and B. Wong.** 2003. An analysis of the *Candida albicans* genome database for soluble secreted proteins using computer-based prediction algorithms. *Yeast* **20:**595–610.
107. **Lenardon, M. D., S. A. Milne, H. M. Mora-Montes, F. A. Kaffarnik, S. C. Peck, A. J. P. Brown, C. A. Munro, and N. A. Gow.** 2010. Phosphorylation regulates polarisation of chitin synthesis in *Candida albicans*. *J. Cell Sci.* **123**(Pt. 1)**:**2199–2206.
108. **Lenardon, M. D., C. A. Munro, and N. A. Gow.** 2010. Chitin synthesis and fungal pathogenesis. *Curr. Opin. Microbiol.* **13:**416–423.
109. **Lenardon, M. D., R. K. Whitton, C. A. Munro, D. Marshall, and N. A. R. Gow.** 2007. Individual chitin synthase enzymes synthesize microfibrils of differing structure at specific locations in *Candida albicans* cell wall. *Mol. Microbiol.* **66:**1164–1173.
110. **Lesage, G., A. M. Sdicu, P. Menard, J. Shapiro, S. Hussein, and H. Bussey.** 2004. Analysis of beta-1,3-glucan assembly in *Saccharomyces cerevisiae* using a synthetic interaction network and altered sensitivity to caspofungin. *Genetics* **167:**35–49.
111. **Lesage, G., J. Shapiro, C. A. Specht, A. M. Sdicu, P. Menard, S. Hussein, A. H. Tong, C. Boone, and H. Bussey.** 2005. An interactional network of genes involved in chitin synthesis in *Saccharomyces cerevisiae*. *BMC Genet.* **6:**8.

112. **Levin, D. E.** 2005. Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **69:** 262–291.
113. **Li, D., D. Williams, D. Lowman, M. A. Monteiro, X. Tan, M. Kruppa, W. Fonzi, E. Roman, J. Pla, and R. Calderone.** 2009. The *Candida albicans* histidine kinase Chk1p: signaling and cell wall mannan. *Fungal Genet. Biol.* **46:**731–741.
114. **Li, F., and S. P. Palecek.** 2003. *EAP1*, a *Candida albicans* gene involved in binding human epithelial cells. *Eukaryot. Cell* **2:**1266–1273.
115. **Li, F., and S. P. Palecek.** 2008. Distinct domains of the *Candida albicans* adhesin Eap1p mediate cell-cell and cell-substrate interactions. *Microbiology* **154:**1193–1203.
116. **Li, F., M. J. Svarovsky, A. J. Karlsson, J. P. Wagner, K. Marchillo, P. Oshel, D. Andes, and S. P. Palecek.** 2007. Eap1p, an adhesin that mediates *Candida albicans* biofilm formation in vitro and in vivo. *Eukaryot. Cell* **6:**931–939.
117. **Li, X. S., M. S. Reddy, D. Baev, and M. Edgerton.** 2003. *Candida albicans* Ssa1/2p is the cell envelope binding protein for human salivary histatin 5. *J. Biol. Chem.* **278:** 28553–28561.
118. **Lopez-Ribot, J. L., M. Casanova, A. Murgui, and J. P. Martinez.** 2004. Antibody response to *Candida albicans* cell wall antigens. *FEMS Immunol. Med. Microbiol.* **41:**187–196.
119. **Lopez-Villar, E., L. Monteoliva, M. R. Larsen, E. Sachon, M. Shabaz, M. Pardo, J. Pla, C. Gil, P. Roepstorff, and C. Nombela.** 2006. Genetic and proteomic evidences support the localization of yeast enolase in the cell surface. *Proteomics* **6**(Suppl. 1)**:S**107–S118.
120. **Lorenz, M. C., J. A. Bender, and G. R. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot. Cell* **3:**1076–1087.
121. **Lott, T. J., B. P. Holloway, D. A. Logan, R. Fundyga, and J. Arnold.** 1999. Towards understanding the evolution of the human commensal yeast *Candida albicans*. *Microbiology* **145:**1137–1143.
122. **Loza, L., Y. Fu, A. S. Ibrahim, D. C. Sheppard, S. G. Filler, and J. E. Edwards, Jr.** 2004. Functional analysis of the *Candida albicans ALS1* gene product. *Yeast* **21:** 473–482.
123. **Lu, C. F., R. C. Montijn, J. L. Brown, F. Klis, J. Kurjan, H. Bussey, and P. N. Lipke.** 1995. Glycosyl phosphatidylinositol-dependent cross-linking of alpha-agglutinin and beta 1,6-glucan in the *Saccharomyces cerevisiae* cell wall. *J. Cell Biol.* **128:**333–340.
124. **MacCallum, D. M., L. Castillo, K. Nather, C. A. Munro, A. J. Brown, N. A. Gow, and F. C. Odds.** 2009. Property differences among the four major *Candida albicans* strain clades. *Eukaryot. Cell* **8:**373–387.
125. **Maddi, A., S. M. Bowman, and S. J. Free.** 2009. Trifluoromethanesulfonic acid-based proteomic analysis of cell wall and secreted proteins of the ascomycetous fungi *Neurospora crassa* and *Candida albicans*. *Fungal Genet. Biol.* **46:**768–781.
126. **Mao, Y., Z. Zhang, C. Gast, and B. Wong.** 2008. C-terminal signals regulate targeting of the GPI-anchored proteins to the cell wall or the plasma membrane in *Candida albicans*. *Eukaryot. Cell* **7:**1906–1915.
127. **Mao, Y., Z. Zhang, and B. Wong.** 2003. Use of green fluorescent protein fusions to analyse the N- and C-terminal signal peptides of GPI-anchored cell wall proteins in *Candida albicans*. *Mol. Microbiol.* **50:**1617–1628.
128. **Marcil, A., C. Gadoury, J. Ash, J. Zhang, A. Nantel, and M. Whiteway.** 2008. Analysis of PRA1 and its relationship to *Candida albicans*-macrophage interactions. *Infect. Immun.* **76:**4345–4358.
129. **Martchenko, M., A. M. Alarco, D. Harcus, and M. Whiteway.** 2004. Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced *SOD5* gene. *Mol. Biol. Cell* **15:**456–467.
130. **Martinez, A. I., L. Castillo, A. Garcera, M. V. Elorza, E. Valentin, and R. Sentandreu.** 2004. Role of Pir1 in the construction of the *Candida albicans* cell wall. *Microbiology* **150:**3151–3161.
131. **Martinez-Lopez, R., L. Monteoliva, R. Diez-Orejas, C. Nombela, and C. Gil.** 2004. The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*. *Microbiology* **150:**3341–3354.
132. **Martin-Yken, H., A. Dagkessamanskaia, F. Basmaji, A. Lagorce, and J. Francois.** 2003. The interaction of Slt2 MAP kinase with Knr4 is necessary for signalling through the cell wall integrity pathway in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **49:**23–35.
133. **Mazan, M., K. Mazanova, and V. Farkas.** 2008. Phenotype analysis of *Saccharomyces cerevisiae* mutants with deletions in Pir cell wall glycoproteins. *Antonie Leeuwenhoek* **94:**335–342.
134. **Mazur, P., and W. Baginsky.** 1996. In vitro activity of 1,3-beta-D-glucan synthase requires the GTP-binding protein Rho1. *J. Biol. Chem.* **271:**14604–14609.
135. **Moreno-Ruiz, E., G. Ortu, P. W. J. de Groot, F. Cottier, C. Loussert, M. C. Prevost, C. de Koster, F. M. Klis, S. Goyard, and C. d'Enfert.** 2009. The GPI-modified proteins Pga59 and Pga62 of *Candida albicans* are required for cell wall integrity. *Microbiology* **155:**2004–2020.
136. **Mormeneo, S., H. Rico, M. Iranzo, C. Aguado, and R. Sentandreu.** 1996. Study of supramolecular structures released from the cell wall of *Candida albicans* by ethylenediamine treatment. *Arch. Microbiol.* **166:**327–335.
137. **Mormeneo, S., R. Zazueta-Sandoval, and A. Flores-Carreon.** 1995. Isolation and partial characterization of uronic acid-containing glycoproteins from *Mucor rouxii*. *Curr. Microbiol.* **30:**237–241.
138. **Moukadiri, I., and J. Zueco.** 2001. Evidence for the attachment of Hsp150/Pir2 to the cell wall of *Saccharomyces cerevisiae* through disulfide bridges. *FEMS Yeast Res.* **1:**241–245.
139. **Mouyna, I., T. Fontaine, M. Vai, M. Monod, W. A. Fonzi, M. Diaquin, L. Popolo, R. P. Hartland, and J. P. Latge.** 2000. Glycosylphosphatidylinositol-anchored glucanosyltransferases play an active role in the biosynthesis of the fungal cell wall. *J. Biol. Chem.* **275:** 14882–14889.
140. **Mouyna, I., W. Morelle, M. Vai, M. Monod, B. Lechenne, T. Fontaine, A. Beauvais, J. Sarfati, M. C. Prevost, C. Henry, and J. P. Latge.** 2005. Deletion of *GEL2* encoding for a beta(1-3)glucanosyltransferase affects morphogenesis and virulence in *Aspergillus fumigatus*. *Mol. Microbiol.* **56:**1675–1688.
141. **Mrsa, V., T. Seidl, M. Gentzsch, and W. Tanner.** 1997. Specific labelling of cell wall proteins by biotinylation. Identification of four covalently linked O-mannosylated proteins of *Saccharomyces cerevisiae*. *Yeast* **13:**1145–1154.
142. **Mrsa, V., and W. Tanner.** 1999. Role of NaOH-extractable cell wall proteins Ccw5p, Ccw6p, Ccw7p and Ccw8p (members of the Pir protein family) in stability of the *Saccharomyces cerevisiae* cell wall. *Yeast* **15:**813–820.
143. **Muhlschlegel, F. A., and W. A. Fonzi.** 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. *Mol. Cell. Biol.* **17:**5960–5967.
144. **Munro, C. A.** 2009. *Candida albicans* cell wall mediated virulence, p. 69–95. *In* R. Ashbee and E. Bignell (ed.),

Handbook of Pathogenic Yeasts. Springer-Verlag, Berlin, Germany.

145. **Munro, C. A., and N. A. Gow.** 2001. Chitin synthesis in human pathogenic fungi. *Med. Mycol.* **39**(Suppl. 1)**:**41–53.
146. **Munro, C. A., D. A. Schofield, G. W. Gooday, and N. A. R. Gow.** 1998. Regulation of chitin synthesis during dimorphic growth of *Candida albicans*. *Microbiology* **144:**391–401.
147. **Munro, C. A., S. Selvaggini, I. de Bruijn, L. Walker, M. D. Lenardon, B. Gerssen, S. Milne, A. J. Brown, and N. A. Gow.** 2007. The PKC, HOG and Ca^{2+} signalling pathways co-ordinately regulate chitin synthesis in *Candida albicans*. *Mol. Microbiol.* **63:**1399–1413.
148. **Naglik, J., A. Albrecht, O. Bader, and B. Hube.** 2004. *Candida albicans* proteinases and host/pathogen interactions. *Cell. Microbiol.* **6:**915–926.
149. **Naglik, J. R., D. Moyes, J. Makwana, P. Kanzaria, E. Tsichlaki, G. Weindl, A. R. Tappuni, C. A. Rodgers, A. J. Woodman, S. J. Challacombe, M. Schaller, and B. Hube.** 2008. Quantitative expression of the *Candida albicans* secreted aspartyl proteinase gene family in human oral and vaginal candidiasis. *Microbiology* **154:** 3266–3280.
150. **Nailis, H., D. Vandenbosch, D. Deforce, H. J. Nelis, and T. Coenye.** 2010. Transcriptional response to fluconazole and amphotericin B in *Candida albicans* biofilms. *Res. Microbiol.* **161:**284–292.
151. **Nantel, A., D. Dignard, C. Bachewich, D. Harcus, A. Marcil, A. P. Bouin, C. W. Sensen, H. Hogues, H. M. van het, P. Gordon, T. Rigby, F. Benoit, D. C. Tessier, D. Y. Thomas, and M. Whiteway.** 2002. Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol. Biol. Cell* **13:**3452–3465.
152. **Nather, K., and C. A. Munro.** 2008. Generating cell surface diversity in *Candida albicans* and other fungal pathogens. *FEMS Microbiol. Lett.* **285:**137–145.
153. **Navarro-Garcia, F., B. Eisman, S. M. Fiuza, C. Nombela, and J. Pla.** 2005. The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*. *Microbiology* **151:**2737–2749.
154. **Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell.** 2006. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation in vitro and in vivo. *PLoS Pathog.* **2:**e63.
155. **Nobile, C. J., H. A. Schneider, J. E. Nett, D. C. Sheppard, S. G. Filler, D. R. Andes, and A. P. Mitchell.** 2008. Complementary adhesin function in *C. albicans* biofilm formation. *Curr. Biol.* **18:**1017–1024.
156. **Nombela, C., C. Gil, and W. L. Chaffin.** 2006. Non-conventional protein secretion in yeast. *Trends Microbiol.* **14:**15–21.
157. **Oh, S. H., G. Cheng, J. A. Nuessen, R. Jajko, K. M. Yeater, X. Zhao, C. Pujol, D. R. Soll, and L. L. Hoyer.** 2005. Functional specificity of *Candida albicans* Als3p proteins and clade specificity of *ALS3* alleles discriminated by the number of copies of the tandem repeat sequence in the central domain. *Microbiology* **151:** 673–681.
158. **Ostrosky-Zeichner, L., B. D. Alexander, D. H. Kett, J. Vazquez, P. G. Pappas, F. Saeki, P. A. Ketchum, J. Wingard, R. Schiff, H. Tamura, M. A. Finkelman, and J. H. Rex.** 2005. Multicenter clinical evaluation of the (1→3) beta-D-glucan assay as an aid to diagnosis of fungal infections in humans. *Clin. Infect. Dis.* **41:**654–659.
159. **Otoo, H. N., K. G. Lee, W. Qiu, and P. N. Lipke.** 2007. *Candida albicans* Als adhesins have conserved amyloid-forming sequences. *Eukaryot. Cell* **7:**776–782.
160. **Pardini, G., P. W. De Groot, A. T. Coste, M. Karababa, F. M. Klis, C. G. De Koster, and D. Sanglard.** 2006. The *CRH* family coding for cell wall glycosylphosphatidylinositol proteins with a predicted transglycosidase domain affects cell wall organization and virulence of *Candida albicans*. *J. Biol. Chem.* **281:**40399–40411.
161. **Perez, A., B. Pedros, A. Murgui, M. Casanova, J. L. Lopez-Ribot, and J. P. Martinez.** 2006. Biofilm formation by *Candida albicans* mutants for genes coding fungal proteins exhibiting the eight-cysteine-containing CFEM domain. *FEMS Yeast Res.* **6:**1074–1084.
162. **Perlin, D. S.** 2007. Resistance to echinocandin-class antifungal drugs. *Drug Resist. Updat.* **10:**121–130.
163. **Pietrella, D., A. Rachini, A. Torosantucci, P. Chiani, A. J. Brown, F. Bistoni, P. Costantino, P. Mosci, C. d'Enfert, R. Rappuoli, A. Cassone, and A. Vecchiarelli.** 2010. A beta-glucan-conjugate vaccine and anti-beta-glucan antibodies are effective against murine vaginal candidiasis as assessed by a novel in vivo imaging technique. *Vaccine* **28:**1717–1725.
164. **Pitarch, A., R. Diez-Orejas, G. Molero, M. Pardo, M. Sanchez, C. Gil, and C. Nombela.** 2001. Analysis of the serologic response to systemic *Candida albicans* infection in a murine model. *Proteomics* **1:**550–559.
165. **Pitarch, A., A. Jimenez, C. Nombela, and C. Gil.** 2006. Decoding serological response to *Candida* cell wall immunome into novel diagnostic, prognostic, and therapeutic candidates for systemic candidiasis by proteomic and bioinformatic analyses. *Mol. Cell. Proteomics* **5:**79–96.
166. **Pitarch, A., M. Pardo, A. Jimenez, J. Pla, C. Gil, M. Sanchez, and C. Nombela.** 1999. Two-dimensional gel electrophoresis as analytical tool for identifying *Candida albicans* immunogenic proteins. *Electrophoresis* **20:**1001–1010.
167. **Pitoniak, A., B. Birkaya, H. M. Dionne, N. Vadaie, and P. J. Cullen.** 2009. The signaling mucins Msb2 and Hkr1 differentially regulate the filamentation mitogen-activated protein kinase pathway and contribute to a multimodal response. *Mol. Biol. Cell* **20:**3101–3114.
168. **Popolo, L., D. Gilardelli, P. Bonfante, and M. Vai.** 1997. Increase in chitin as an essential response to defects in assembly of cell wall polymers in the *ggp1*Δ mutant of *Saccharomyces cerevisiae*. *J. Bacteriol.* **179:**463–469.
169. **Popolo, L., E. Ragni, C. Carotti, O. Palomares, R. Aardema, J. W. Back, H. L. Dekker, L. J. de Koning, L. de Jong, and C. G. De Koster.** 2008. Disulfide bond structure and domain organization of yeast beta(1,3)-glucanosyltransferases involved in cell wall biogenesis. *J. Biol. Chem.* **283:**18553–18565.
170. **Popolo, L., and M. Vai.** 1999. The Gas1 glycoprotein, a putative wall polymer cross-linker. *Biochim. Biophys. Acta* **1426:**385–400.
171. **Qadota, H., C. P. Python, S. B. Inoue, M. Arisawa, Y. Anraku, Y. Zheng, T. Watanabe, D. E. Levin, and Y. Ohya.** 1996. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. *Science* **272:**279–281.
172. **Ragni, E., A. Coluccio, E. Rolli, J. M. Rodriguez-Pena, G. Colasante, J. Arroyo, A. M. Neiman, and L. Popolo.** 2007. *GAS2 a*nd *GAS4*, a pair of developmentally regulated genes required for spore wall assembly in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **6:**302–316.
173. **Ragni, E., T. Fontaine, C. Gissi, J. P. Latge, and L. Popolo.** 2007. The Gas family of proteins of *Saccharomyces cerevisiae*: characterization and evolutionary analysis. *Yeast* **24:**297–308.
174. **Ramsook, C. B., C. Tan, M. C. Garcia, R. Fung, G. Soybelman, R. Henry, A. Litewka, S. O'Meally, H. N. Otoo, R. A. Khalaf, A. M. Dranginis, N. K. Gaur, S. A.**

Klotz, J. M. Rauceo, C. K. Jue, and P. N. Lipke. 2010. Yeast cell adhesion molecules have functional amyloid-forming sequences. *Eukaryot. Cell* **9:**393–404.
175. **Rauceo, J. M., R. Dearmond, H. Otoo, P. C. Kahn, S. A. Klotz, N. K. Gaur, and P. N. Lipke.** 2006. Threonine-rich repeats increase fibronectin binding in the *C. albicans* adhesin Als5p. *Eukaryot. Cell* **5:**1664–1673.
176. **Reid, D. M., N. A. Gow, and G. D. Brown.** 2009. Pattern recognition: recent insights from Dectin-1. *Curr. Opin. Immunol.* **21:**30–37.
177. **Richard, M., S. Ibata-Ombetta, F. Dromer, F. Bordon-Pallier, T. Jouault, and C. Gaillardin.** 2002. Complete glycosylphosphatidylinositol anchors are required in *Candida albicans* for full morphogenesis, virulence and resistance to macrophages. *Mol. Microbiol.* **44:**841–853.
178. **Richard, M. L., and A. Plaine.** 2007. Comprehensive analysis of glycosylphosphatidylinositol-anchored proteins in *Candida albicans*. *Eukaryot. Cell* **6:**119–133.
179. **Ripeau, J. S., M. Fiorillo, F. Aumont, P. Belhumeur, and L. De Repentigny.** 2002. Evidence for differential expression of *Candida albicans* virulence genes during oral infection in intact and human immunodeficiency virus type 1-transgenic mice. *J. Infect. Dis.* **185:**1094–1102.
180. **Rodriguez-Pena, J. M., V. J. Cid, J. Arroyo, and C. Nombela.** 2000. A novel family of cell wall-related proteins regulated differently during the yeast life cycle. *Mol. Cell. Biol.* **20:**3245–3255.
181. **Rodriguez-Pena, J. M., C. Rodriguez, A. Alvarez, C. Nombela, and J. Arroyo.** 2002. Mechanisms for targeting of the *Saccharomyces cerevisiae* GPI-anchored cell wall protein Crh2p to polarised growth sites. *J. Cell Sci.* **115:**2549–2558.
182. **Roman, E., R. Alonso-Monge, Q. Gong, D. Li, R. Calderone, and J. Pla.** 2009. The Cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen *Candida albicans*. *FEMS Yeast Res.* **9:**942–955.
183. **Roman, E., F. Cottier, J. F. Ernst, and J. Pla.** 2009. Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in *Candida albicans*. *Eukaryot. Cell* **8:**1235–1249.
184. **Russo, P., N. Kalkkinen, H. Sareneva, J. Paakkola, and M. Makarow.** 1992. A heat shock gene from *Saccharomyces cerevisiae* encoding a secretory glycoprotein. *Proc. Natl. Acad. Sci. USA* **89:**3671–3675.
185. **Sandini, S., R. La Valle, F. De Bernardis, C. Macri, and A. Cassone.** 2007. The 65 kDa mannoprotein gene of *Candida albicans* encodes a putative beta-glucanase adhesin required for hyphal morphogenesis and experimental pathogenicity. *Cell. Microbiol.* **9:**1223–1238.
186. **Saporito-Irwin, S. M., C. E. Birse, P. S. Sypherd, and W. A. Fonzi.** 1995. *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. *Mol. Cell. Biol.* **15:**601–613.
187. **Sarthy, A. V., T. McGonigal, M. Coen, D. J. Frost, J. A. Meulbroek, and R. C. Goldman.** 1997. Phenotype in *Candida albicans* of a disruption of the *BGL2* gene encoding a 1,3-β-glucosyltransferase. *Microbiology* **143:**367–376.
188. **Schaller, M., U. Boeld, S. Oberbauer, G. Hamm, B. Hube, and H. C. Korting.** 2004. Polymorphonuclear leukocytes (PMNs) induce protective Th1-type cytokine epithelial responses in an in vitro model of oral candidosis. *Microbiology* **150:**2807–2813.
189. **Senn, L., J. O. Robinson, S. Schmidt, M. Knaup, N. Asahi, S. Satomura, S. Matsuura, B. Duvoisin, J. Bille, T. Calandra, and O. Marchetti.** 2008. 1,3-Beta-D-glucan antigenemia for early diagnosis of invasive fungal infections in neutropenic patients with acute leukemia. *Clin. Infect. Dis.* **46:**878–885.
190. **Shahinian, S., and H. Bussey.** 2000. β-1,6-Glucan synthesis in *Saccharomyces cerevisiae*. *Mol. Microbiol.* **35:**477–489.
191. **Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu, A. S. Ibrahim, S. G. Filler, M. Zhang, A. J. Waring, and J. E. Edwards, Jr.** 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. *J. Biol. Chem.* **279:**30480–30489.
192. **Sosinska, G. J., P. W. De Groot, M. J. Teixeira de Mattos, H. L. Dekker, C. G. De Koster, K. J. Hellingwerf, and F. M. Klis.** 2008. Hypoxic conditions and iron restriction affect the cell-wall proteome of *Candida albicans* grown under vagina-simulative conditions. *Microbiology* **154:**510–520.
193. **Spellberg, B. J., A. S. Ibrahim, V. Avanesian, Y. Fu, C. Myers, Q. T. Phan, S. G. Filler, M. R. Yeaman, and J. E. Edwards, Jr.** 2006. Efficacy of the anti-*Candida* rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J. Infect. Dis.* **194:**256–260.
194. **Spreghini, E., D. A. Davis, R. Subaran, M. Kim, and A. P. Mitchell.** 2003. Roles of *Candida albicans* Dfg5p and Dcw1p cell surface proteins in growth and hypha formation. *Eukaryot. Cell* **2:**746–755.
195. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283:**1535–1538.
196. **Staab, J. F., C. A. Ferrer, and P. Sundstrom.** 1996. Developmental expression of a tandemly repeated, proline and glutamine rich amino acid motif on hyphal surfaces of *Candida albicans*. *J. Biol. Chem.* **271:**6298–6305.
197. **Sullivan, P. A., C. Y. Yin, C. Molloy, M. D. Templeton, and M. G. Shepherd.** 1983. An analysis of the metabolism and cell wall composition of *Candida albicans* during germ tube formation. *Can. J. Microbiol.* **29:**1514–1525.
198. **Takeda, J., and T. Kinoshita.** 1995. GPI-anchor biosynthesis. *Trends Biochem. Sci.* **20:**367–371.
199. **Tatebayashi, K., K. Tanaka, H. Y. Yang, K. Yamamoto, Y. Matsushita, T. Tomida, M. Imai, and H. Saito.** 2007. Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the *SHO1* branch of yeast HOG pathway. *EMBO J.* **26:**3521–3533.
200. **Teparic, R., I. Stuparevic, and V. Mrsa.** 2007. Binding assay for incorporation of alkali-extractable proteins in the *Saccharomyces cerevisiae* cell wall. *Yeast* **24:**259–266.
201. **Theiss, S., G. Ishdorj, A. Brenot, M. Kretschmar, C. Y. Lan, T. Nichterlein, J. Hacker, S. Nigam, N. Agabian, and G. A. Kohler.** 2006. Inactivation of the phospholipase B gene PLB5 in wild-type *Candida albicans* reduces cell-associated phospholipase A2 activity and attenuates virulence. *Int. J. Med. Microbiol.* **296:**405–420.
202. **Tiede, A., I. Bastisch, J. Schubert, P. Orlean, and R. E. Schmidt.** 1999. Biosynthesis of glycosylphosphatidylinositols in mammals and unicellular microbes. *Biol. Chem.* **380:**503–523.
203. **Toh-e, A., S. Yasunaga, H. Nisogi, K. Tanaka, T. Oguchi, and Y. Matsui.** 1993. Three yeast genes, *PIR1*, *PIR2* and *PIR3*, containing internal tandem repeats, are related to each other, and *PIR1* and *PIR2* are required for tolerance to heat shock. *Yeast* **9:**481–494.
204. **Torosantucci, A., M. J. Gomez, C. Bromuro, I. Casalinuovo, and A. Cassone.** 1991. Biochemical and antigenic characterization of mannoprotein constituents released from yeast and mycelial forms of *Candida albicans*. *J. Med. Vet. Mycol.* **29:**361–372.
205. **Umeyama, T., A. Kaneko, H. Watanabe, A. Hirai, Y. Uehara, M. Niimi, and M. Azuma.** 2006. Deletion of the *CaBIG1* gene reduces β-1,6-glucan synthesis, filamenta-

tion, adhesion, and virulence in *Candida albicans*. *Infect. Immun.* **74:**2373–2381.
206. **Vadaie, N., H. Dionne, D. S. Akajagbor, S. R. Nickerson, D. J. Krysan, and P. J. Cullen.** 2008. Cleavage of the signaling mucin Msb2 by the aspartyl protease Yps1 is required for MAPK activation in yeast. *J. Cell Biol.* **181:**1073–1081.
207. **Valdivieso, M. H., L. Ferrario, M. Vai, A. Duran, and L. Popolo.** 2000. Chitin synthesis in a *gas1* mutant of *Saccharomyces cerevisiae*. *J. Bacteriol.* **182:**4752–4757.
208. **Verstrepen, K. J., and G. R. Fink.** 2009. Genetic and epigenetic mechanisms underlying cell-surface variability in protozoa and fungi. *Annu. Rev. Genet.* **43:**1–24.
209. **Verstrepen, K. J., A. Jansen, F. Lewitter, and G. R. Fink.** 2005. Intragenic tandem repeats generate functional variability. *Nat. Genet.* **37:**986–990.
210. **Walker, L. A., N. A. Gow, and C. A. Munro.** 2009. Fungal echinocandin resistance. *Fungal Genet. Biol.* **47:**117–126.
211. **Walker, L. A., C. A. Munro, I. de Bruijn, M. D. Lenardon, A. McKinnon, and N. A. R. Gow.** 2008. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. *PLoS Pathog.* **4:**e1000040.
212. **Weig, M., K. Haynes, T. R. Rogers, O. Kurzai, M. Frosch, and F. A. Muhlschlegel.** 2001. A GAS-like gene family in the pathogenic fungus *Candida glabrata*. *Microbiology* **147:**2007–2019.
213. **Weissman, Z., and D. Kornitzer.** 2004. A family of *Candida* cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. *Mol. Microbiol.* **53:**1209–1220.
214. **Weissman, Z., R. Shemer, E. Conibear, and D. Kornitzer.** 2008. An endocytic mechanism for haemoglobin-iron acquisition in *Candida albicans*. *Mol. Microbiol.* **69:**201–217.
215. **Wheeler, R. T., D. Kombe, S. D. Agarwala, and G. R. Fink.** 2008. Dynamic, morphotype-specific *Candida albicans* beta-glucan exposure during infection and drug treatment. *PLoS Pathog.* **4:**e1000227.
216. **Xin, H., S. Dziadek, D. R. Bundle, and J. E. Cutler.** 2008. Synthetic glycopeptide vaccines combining beta-mannan and peptide epitopes induce protection against candidiasis. *Proc. Natl. Acad. Sci. USA* **105:**13526–13531.
217. **Yabe, T., T. Yamada-Okabe, S. Kasahara, Y. Furuichi, T. Nakajima, E. Ichishima, M. Arisawa, and H. Yamada-Okabe.** 1996. *HKR1* encodes a cell surface protein that regulates both cell wall beta-glucan synthesis and budding pattern in the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* **178:**477–483.
218. **Yi, S., N. Sahni, K. J. Daniels, C. Pujol, T. Srikantha, and D. R. Soll.** 2008. The same receptor, G protein, and mitogen-activated protein kinase pathway activate different downstream regulators in the alternative white and opaque pheromone responses of *Candida albicans*. *Mol. Biol. Cell* **19:**957–970.
219. **Yin, Q. Y., P. W. De Groot, C. G. De Koster, and F. M. Klis.** 2008. Mass spectrometry-based proteomics of fungal wall glycoproteins. *Trends Microbiol.* **16:**20–26.
220. **Yin, Q. Y., P. W. J. De Groot, H. L. Dekker, L. de Jong, F. M. Klis, and C. G. De Koster.** 2005. Comprehensive proteomic analysis of *Saccharomyces cerevisiae* cell walls: identification of proteins covalently attached via glycosylphosphatidylinositol remnants or mild alkali-sensitive linkages. *J. Biol. Chem.* **280:**20894–20901.
221. **Yun, D. J., Y. Zhao, J. M. Pardo, M. L. Narasimhan, B. Damsz, H. Lee, L. R. Abad, M. P. D'Urzo, P. M. Hasegawa, and R. A. Bressan.** 1997. Stress proteins on the yeast cell surface determine resistance to osmotin, a plant antifungal protein. *Proc. Natl. Acad. Sci. USA* **94:**7082–7087.
222. **Zhang, N., A. L. Harrex, B. R. Holland, L. E. Fenton, R. D. Cannon, and J. Schmid.** 2003. Sixty alleles of the *ALS7* open reading frame in *Candida albicans: ALS7* is a hypermutable contingency locus. *Genome Res.* **13:**2005–2017.
223. **Zhao, X., S. H. Oh, and L. L. Hoyer.** 2007. Unequal contribution of *ALS9* alleles to adhesion between *Candida albicans* and human vascular endothelial cells. *Microbiology* **153:**2342–2350.
224. **Zhao, X., C. Pujol, D. R. Soll, and L. L. Hoyer.** 2003. Allelic variation in the contiguous loci encoding *Candida albicans ALS5, ALS1* and *ALS9*. *Microbiology* **149:**2947–2960.
225. **Zupancic, M. L., M. Frieman, D. Smith, R. A. Alvarez, R. D. Cummings, and B. P. Cormack.** 2008. Glycan microarray analysis of *Candida glabrata* adhesin ligand specificity. *Mol. Microbiol.* **68:**547–559.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch15

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Stress Responses in *Candida*

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For many years, many groups have investigated the virulence factors of *Candida albicans* and their contributions to pathogenicity and the infection process (22, 26, 27, 134, 135). More recently, attention has also turned to those fitness attributes that contribute to the physiological robustness of *C. albicans* in host niches. These fitness attributes include metabolic adaptability, which promotes the effective assimilation of a wide range of alternative nutrients (19, 21, 99, 141), and any other phenotypic trait that promotes survival as opposed to disease during growth in vivo. Fitness attributes also encompass the robust stress responses that contribute to fungal pathogenicity by facilitating the adaptation of the pathogen to its niche within the host and by helping to protect the fungal invader against the immunological defenses of the host. This chapter briefly summarizes our current understanding of stress responses in *Candida albicans*. The focus is on "natural" stresses rather than the "artificial" imposed stresses imposed by antifungal drugs, for example, which are covered elsewhere in this volume.

Of all the pathogenic *Candida* species, the stress responses of *C. albicans* have been investigated in the greatest depth. Where appropriate, mention is made of studies with other pathogenic *Candida* species. However, so far, relatively few studies have been performed on pathogenic *Candida* species other than *C. albicans*. Nevertheless, much is likely to be gained by studying stress regulation in these diverse species. Comparisons of stress responses in *C. albicans* with those of its benign cousins *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have revealed fundamental similarities in the protective mechanisms that are activated (131). For example, osmolyte production is activated in response to osmotic stress, whereas redox protection and detoxification mechanisms are stimulated in response to oxidative or nitrosative stresses. Furthermore, core regulatory modules are evolutionarily conserved among *C. albicans*, *S. cerevisiae*, and *S. pombe*. For example, the stress-activated protein kinases Hog1 (in *S. cerevisiae* and *C. albicans*) and Sty1 (in *S. pombe*) play central roles in the osmotic stress response, whereas the heat shock transcription factor Hsf1 is critical for adaptation to thermal stress. Interestingly, while these regulatory modules have been conserved, in some cases their cellular roles have diverged and become tailored to different contexts that define the niche of different organisms. Presumably this divergence has arisen because these yeasts have routinely been exposed to differing types and amplitudes of stress during the course of their evolution in contrasting niches.

This is particularly interesting from a systems biology perspective. Indeed, a complete understanding of stress responses and their impact upon pathogenicity depends upon the elaboration of the networks that mediate these responses and the dynamic behaviors of these networks. Furthermore, comparisons of stress networks and their dynamic behaviors in divergent species such as *C. albicans*, *S. cerevisiae*, and *S. pombe* are likely to be particularly revealing. Such comparisons will provide important insights into the fundamental requirements of a robust stress response and hence into the selective pressures that have been imposed during the evolution of these contemporary yeasts from their ancient common ancestor. Such comparisons will also raise questions as to how dynamically robust stress responses were maintained during the evolution of this ancestral yeast into these divergent contemporary ascomycetes, such as pathogenic *Candida* species and benign model yeasts. These systems biology-related questions will be the focus of future studies and reviews. The focus of this review is stress responses in *C. albicans* and their role in pathogenicity.

CANDIDA ALBICANS STRESS RESPONSES IN THE CONTEXT OF HOST NICHES

Where is *C. albicans* found? *C. albicans* is frequently described as a major fungal pathogen of humans, but it is frequently isolated from healthy individuals as well as from patients. *C. albicans* has also been isolated from mammals and other animals. In contrast, *C. albicans* is rarely isolated from the environment, and these isolates can often be attributed to contamination by humans or animals. Hence, this fungus is considered to be obligately associated with warm-blooded animals (134).

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What niches does *C. albicans* occupy in humans? As described in the last section of this volume, the majority of healthy individuals carry *C. albicans* as a relatively harmless commensal in the microbial flora of their gastrointestinal or urogenital tract (26, 134). When their microbial flora is disturbed or host defenses become compromised, *C. albicans* can cause mucocutaneous infections of the oral cavity, gastrointestinal tract, and urogenital tract. While most of these infections can be treated successfully using antifungal drugs, a small proportion of these infections can be recurrent, the basis of these recurrent infections probably relating to the immune status of the patient (59, 60). Systemic infections can arise in severely immunocompromised patients. When the immunological defenses of the host are lowered, *C. albicans* can survive in the bloodstream and colonize internal organs such as the kidneys, spleen, liver, and brain. Since most *C. albicans* infections, whether superficial or systemic, seem to arise mainly as a result of changes in the status of the host rather than the fungus, the prevailing view is that *C. albicans* is an opportunistic pathogen that has evolved primarily as a commensal organism of warm-blooded animals (26, 134). Therefore, it would seem most appropriate to consider the stress responses of *C. albicans* in the context of commensalism rather than virulence. However, given the clinical importance of this fungus, it is hardly surprising that most research on *C. albicans* has been directed towards an understanding of this organism as a pathogen and the treatment of *Candida* infections. This is also true for experiments that have addressed the stress responses of this organism.

What stresses might *C. albicans* encounter in its natural environment? The exact nature of the multifarious microenvironments that *C. albicans* occupies in its mammalian host has not been established. Hence, the nature of the stresses that this pathogen encounters in vivo has not been defined in any detail. Nevertheless, *C. albicans* is exposed to oxidative and nitrosative stresses when it encounters the innate immune system (see below). Furthermore, the fungus is probably exposed to osmotic stress in the kidneys and in the oral cavity and to pH stress in the gastrointestinal and urogenital tracts. Certainly there will be less uniformity between the stress responses encountered in vivo and the experimental conditions used in vitro to dissect certain stress responses. For example, experimental analyses of stress responses tend to exploit dramatic and acute stress doses to reveal large and measurable molecular or cellular phenotypes. In certain cases this might be relevant to the in vivo situation, for example, the sudden exposure to reactive oxygen and nitrogen species following phagocytosis. On the other hand, dramatic temperature upshifts of 12 to 15°C are often employed to investigate the heat shock response, and large doses of NaCl are often used to study osmotic stress responses. In reality these stress responses have probably evolved to maintain thermal and osmotic homeostasis in the face of less dramatic changes in the microenvironment, for example, during the relatively slow temperature changes of the febrile host or more subtle changes in osmolarity.

The above discussion implies that stress responses are important for the survival of *C. albicans* in its host. Is this the case? The data indicate that the regulatory pathways that control stress responses, as well as the downstream targets of these pathways, are required for the full virulence of *C. albicans*. For example, the inactivation of genes encoding enzymes that detoxify reactive oxygen species (catalase, Cat1; superoxide dismutases, Sod1 and Sod5; and thioredoxin, Trx1) or reactive nitrogen species (the Yhb1 hemoprotein), or trehalose biosynthetic enzymes (Tps1 and Tps2), have all been reported to attenuate the virulence of *C. albicans* in the mouse model of systemic candidiasis (43, 62, 84, 86, 107, 188, 190, 204, 209). Similarly, the deletion of the gene encoding the stress-activated protein kinase (SAPK) Hog1 decreases the virulence of *C. albicans* cells (5). These data strongly suggest that stress responses promote survival of this fungus in the host, at least during systemic infections.

CELLULAR RESPONSES TO STRESSES IN *C. ALBICANS*

Heat Shock Response

Heat shock was one of the first stresses to be examined in *C. albicans*. Zeuthen and Howard (210) showed that *C. albicans* induces the expression of heat shock proteins (HSPs) following exposure to temperatures of >40°C, and that the expression of these proteins correlates with the induction of thermotolerance in *C. albicans*. More recent genome-wide studies of the heat shock transcriptome in *C. albicans* have confirmed that this pathogen activates the expression of *HSP* genes in response to heat shock (50) and that this transcriptional response to heat shock is regulated largely by the heat shock transcription factor Hsf1 (129). In other organisms, such HSPs have been shown to protect cells by acting as molecular chaperones. Chaperone function has been confirmed for at least one *C. albicans* HSP (137). Some of these chaperones, such as Hsp90, are essential for viability, and Hsf1 is required for their expression even in the absence of heat shock (129, 167, 180). Not surprisingly, therefore, Hsf1 is essential in *C. albicans* (129). Also, Hsf1 activation is required for the full virulence of *C. albicans* in the mouse model of systemic infection (130).

S. cerevisiae and *S. pombe* also respond to heat shock by accumulating trehalose, which is thought to provide some protection against thermal stresses. Likewise, *C. albicans* accumulates trehalose in response to a heat shock (14). However, trehalose biosynthetic genes do not appear to be activated under equivalent conditions (50, 53, 129), possibly because trehalose accumulation might be regulated at a posttranscriptional level in *C. albicans* (63).

The expression of HSPs in *C. albicans* has received special attention for at least two reasons. Firstly, HSPs are induced during the yeast-to-hypha transition (178, 210), which has special significance with respect to the virulence of *C. albicans*. This induction of *HSP* gene expression correlates with the temperature upshifts often associated with the stimulation of hyphal development in vitro (179, 180). Nevertheless, elegant studies by Leah Cowen's group have now shown that Hsp90 plays a direct role in morphogenetic regulation by inhibiting a component of the Ras-cyclic AMP (Ras-cAMP) signaling pathway (167). Thus, elevated temperature tends to relieve the repression of this pathway, thereby promoting hypha formation at body temperature. Secondly, *C. albicans* HSPs are immunogenic in patients (110, 177, 178). Furthermore, immunization with Hsp70 sensitizes animals to systemic *Candida* infection, reducing their survival time by hyperactivating their immune systems (20). In contrast, antibodies against Hsp90 appear to provide immunoprotection against systemic candidiasis (109, 111, 112), and hence, there has been much interest in the

diagnostic and therapeutic applications of anti-Hsp90 antibodies (113).

Osmotic Stress Response

Considerable efforts have yielded a fairly detailed understanding of osmotic stress responses and the mechanisms by which they are regulated in *S. cerevisiae* (reviewed in reference 82). Hyperosmotic stress is usually imposed by treatment with salt or sorbitol. The responses to salt and sorbitol are similar, but Na^+ is toxic (82), and therefore, global responses to NaCl include detoxification functions as well as osmoregulatory functions.

When yeast cells encounter a hyperosmotic shock, this causes rapid water loss from the cell, and as a result turgor pressure is lost and the cell shrinks. The yeast cell must respond by restoring turgor pressure before it can resume growth. This is achieved by the intracellular accumulation of the osmolyte glycerol, by both increasing glycerol synthesis and promoting glycerol accumulation by closing the glyceroaquaporin Fps1 (101, 181). Glycerol synthesis is increased in budding yeast by the transcriptional activation of genes encoding glycerol-3-phosphate dehydrogenase (*GPD1*) and glycerol-3-phosphatase (*GPP1/RHR2* and *GPP2*) (3, 11, 81, 132, 136).

Water rapidly enters the budding yeast cell following a hypo-osmotic stress, causing a marked increase in turgor pressure and cell expansion. This results in the rapid opening of the glyceroaquaporin Fps1, thereby allowing glycerol to escape from the cell and turgor pressure to return to normal (101, 181). Glycerol export is almost entirely dependent upon Fps1. Adaptation to hypo-osmotic stress is rapid, whereas adaptation to hyperosmotic stress is relatively slow, requiring gene activation and glycerol accumulation.

Our understanding of osmotic stress responses in *C. albicans* is less complete. Nevertheless, they appear to be similar to those in *S. cerevisiae*. *C. albicans* accumulates glycerol in response to osmotic stress, and mutations that block glycerol accumulation confer sensitivity to osmotic stress (55, 89, 162). Also, microarray studies of the transcriptional response to osmotic stress have revealed that *C. albicans* induces glycerol biosynthetic enzymes in response to osmotic stress (50, 51). Presumably a glyceroaquaporin plays a key role in the adaptation of *C. albicans* to osmotic stresses, but to our knowledge this has not been studied, and no Fps1 homologue has been annotated in the *Candida* Genome Database or CandidaDB (158, 171).

Osmotic stress responses in *S. cerevisiae* have been the target of numerous mathematical modeling studies. These models range from relatively low-granularity models that examine the structure of the whole cellular system or parts thereof (e.g., see references 79 and 121) to more detailed models that describe the quantitative behaviors of many components of the system (e.g., see reference 90). These models are providing valuable insights into dynamic aspects of osmotic adaptation in budding yeast. Arguably one of the most valuable outputs is the renewed appreciation that the primary function of this system is the homeostatic maintenance of water balance in response to constantly changing conditions in the microenvironment, rather than survival in the face of sudden exposure to 1 M NaCl in a shake flask. Although *C. albicans* and *C. glabrata* are more resistant to salt stress than *S. cerevisiae* (131), this principle almost certainly holds true for these pathogenic yeasts, suggesting that osmotic stress adaptation underpins the physiological robustness of these pathogens in the host niches they occupy. Interestingly, although *Candida dubliniensis* is closely related to *C. albicans*, *C. dubliniensis* is more sensitive to salt stress than *C. albicans* (10, 142). This salt sensitivity appears to be due to a defect in a salt detoxification function, notably the sodium ion transporter Ena21, in *C. dubliniensis* (53).

Oxidative Stress Response

Aerobic yeasts are exposed to reactive oxygen species that are generated as a by-product of oxidative metabolism. Pathogenic fungi must cope with these toxic metabolites and must also cope with the oxidative burst generated by phagocytic cells of the host immune system. These reactive oxygen species include peroxide, superoxide anions, and hydroxyl radicals, which cause significant damage to nucleic acids, proteins, and lipids. Fungi react to oxidative stresses in three main ways: they generate antioxidants that counteract the reactive oxygen species, they produce enzymes that detoxify the reactive oxygen species, and they repair the damage caused by the reactive oxygen species. In particular, catalases and superoxide dismutases are vital for the metabolic detoxification of reactive oxygen species. Also, the glutathione/glutaredoxin and thioredoxin systems play critical roles in repairing oxidatively damaged proteins, protein (re)folding, and sulfur metabolism (72). High doses of peroxide stress stimulate programmed cell death in *C. albicans*, a process that is accelerated by Ras-cAMP signaling (139, 140).

These cellular adaptations to oxidative stress are reflected in the global transcriptional responses of yeasts to such stresses. For example, *S. cerevisiae* responds by activating the transcription of genes involved in glutathione biosynthesis, superoxide dismutase and glutathione peroxidase genes, and genes involved in oxidation and reduction reactions (e.g., thioredoxin, thioredoxin reductases, glutaredoxin, and glutathione reductase) (34, 66). Similarly, catalase, peroxidase, and thioredoxin reductase genes are transcriptionally induced in *S. pombe* in response to H_2O_2 (39).

C. albicans and *C. glabrata* are much more resistant to oxidative stresses than *S. pombe* and *S. cerevisiae* (88, 131). Nevertheless, these yeasts generally display similar transcriptional responses to oxidative stresses (50, 51, 153). *C. glabrata* induces the expression of core oxidative stress response genes, such as *TRR1*, *TRX1*, *CTA1*, *SOD1*, and *GPX1*, in response to low levels of peroxide (153). In *C. albicans*, glutathione biosynthesis (*GSH1*), catalase (*CTA1*), glutathione reductase (*TTR1*), thioredoxin (*TRX1*), and superoxide dismutase (*SOD2*) genes are induced in response to H_2O_2 (50, 51). Therefore, antioxidant biosynthesis, detoxification enzymes, and damage limitation appear to play important roles in the responses of both pathogens to oxidative stresses.

This view is supported by phenotypic analyses of mutants with defects in specific stress genes. For example, catalase is essential for oxidative stress resistance in *C. glabrata* (42). Also in *C. albicans*, defects in a catalase (Cta1), superoxide dismutases (Sod1, Sod4, and Sod5), and trehalose biosynthesis (Tps1) all increase the sensitivity of *C. albicans* cells to oxidative stresses (9, 62, 64, 68, 86, 107, 204). The loss of mitochondrial membrane potential, through Goa1 inactivation, has also been associated with oxidative stress sensitivity in *C. albicans* (15). Interestingly, many of these mutations also attenuate the virulence of *C. albicans* in the mouse model of systemic candidiasis. Furthermore, the inactivation of Sod4 and Sod5, which are expressed at the cell surface, render *C. albicans* more susceptible to killing by the

oxidative bursts of host immune defenses (62, 64). The thiol-specific antioxidant Tsa1 is also expressed at the cell surface (189), and the response regulator Ssk1 appears to coordinate the expression of oxidative stress functions with some cell wall genes (35). Therefore, *C. albicans* has evolved extracellular as well as intracellular mechanisms for protection against reactive oxygen species.

In addition to oxidative stress-stimulated induction of both intracellular and extracellular antioxidants, exposure of *C. albicans* to hydrogen peroxide and other genotoxic stress-inducing agents stimulates morphogenesis (43, 123, 168). In particular, oxidative stress induces the formation of hyperpolarized buds, a filamentous form that is distinct from both hyphae and pseudohyphae. Consistent with this, hyperpolarized bud formation occurs independently of the hyphal regulators Efg1 and Cph1 and instead depends on activation of the Rad53 DNA damage checkpoint pathway (43, 168). A recent study revealed that H_2O_2-induced oxidation, and thus inactivation of the antioxidant thioredoxin protein Trx1, triggers activation of Rad53, which, in turn, stimulates polarized cell growth (43).

Studies with fission yeast indicated that responses to oxidative stress are dose dependent (147). A similar situation probably exists in *C. albicans*, because this pathogen appears to display different transcriptional responses to relatively low and high H_2O_2 concentrations (0.4 and 5 mM, respectively) (50, 51). Some sets of genes were differentially induced in response to 0.4 and 5 mM H_2O_2. For example, a set of genes involved in carbohydrate metabolism (*ICL1*, *GPM2*, *GSY1*, *MLS1*, *NTH1*, and *PCK1*) were induced only in response to high levels of peroxide stress, whereas genes involved in the DNA damage response (*HNT2*, *IPF4708*, *IPF4356*, and *RGA2*) were activated specifically in response to low levels of H_2O_2. Also, genes involved in chromatin silencing and epigenetic regulation (*DOT4*, *DOT6*, *IPF9787*, *ISW2*, and *SAS10*) were specifically repressed in response to high H_2O_2 concentrations. However, other *C. albicans* genes responded to both low and high H_2O_2 concentrations, such as those involved in peroxide detoxification (*CAP1*, *CTA1*, *GPX1*, *GST3*, *TRR1*, and *TRX1*) (51).

These observations have obvious implications in vivo, depending upon the local dose of oxidative stress that *C. albicans* is exposed to. Transcript profiling studies have indicated that the oxidative stress response is activated when *C. albicans* cells are exposed to phagocytic cells (61, 62, 100, 159). Also, microarray experiments suggest that some oxidative stress genes are upregulated in *C. albicans* following exposure to mucosal tissue (208), but such responses are less apparent in the *C. albicans* transcriptome during peritoneal or renal infections (184, 197). Analyses of green fluorescent protein fusions in ex vivo and in vivo infection models have reinforced these observations. The oxidative stress response is activated following phagocytosis by macrophages or neutrophils, but it does not appear to be active in *C. albicans* cells that have established systemic kidney infections (52).

Nitrosative Stress Response

Not so much attention has been paid to nitrosative stress responses in *C. albicans*, and yet these appear to be particularly significant in the context of infection because neutrophils generate reactive nitrogen species (such as $NO^{\cdot}$ radicals) that contribute to their ability to kill *C. albicans* (49, 151, 191). Indeed, reactive nitrogen species may act synergistically with reactive oxygen species in microbial killing (56). As might be expected, microarray analyses have confirmed the activation of nitrosative stress responses following attack by phagocytic cells (62, 208). Furthermore, nitrosative stress genes are upregulated during invasive infections of the mucosal epithelium (208). However, nitrosative stress responses appear to be less apparent in the *C. albicans* transcriptome during peritoneal or renal infections (184, 197).

The roles of the *C. albicans* flavohemoglobin-related proteins Yhb1, Yhb4, and Yhb5 in nitric oxide defenses have been examined (188). The authors reasoned that these proteins might play a protective role because bacterial and yeast nitric oxide dioxygenases (flavohemoproteins) detoxify nitric oxide by converting it to nitrate. While *S. cerevisiae* contains a single Yhb homologue, Yhb1 (213), *C. albicans* contains three: Yhb1, Yhb4, and Yhb5 (188). However, only Yhb1 appears to be required for protection against reactive nitrogen species. *YHB1* expression is induced in response to nitric oxide (84, 188), and *YHB1* inactivation renders *C. albicans* cells sensitive to nitric oxide (188). Furthermore, the virulence of *C. albicans yhb1* cells is attenuated compared with that of wild-type controls in the mouse model of systemic candidiasis (84). However, Hromatka and coworkers suggest that nitric oxide production is not a prime determinant of virulence in this mouse model, because the virulence defect of *C. albicans yhb1* cells is not suppressed by the inactivation of the *NOS2* (inducible nitric oxide synthase) gene in the mouse host. It is conceivable that the hyperfilamentous phenotype of *yhb1* cells might account for their virulence defect, rather than their defect in nitric oxide detoxification (84). Therefore, further experiments are required to test the idea that reactive nitrogen species play an important role in protecting the host against *Candida* infection. Interestingly, *C. albicans* yeast cells may be more resistant to reactive nitrogen species than hyphal cells (16), but the molecular basis for this has not been established.

Microarray analyses of the nitrosative stress response in *C. albicans* revealed the activation of 65 genes, many of which have been implicated in the response to reactive oxygen species (84). These include catalase (*CTA1*), glutathione-conjugating and -modifying enzymes, and NADPH oxidoreductases and dehydrogenases. Therefore, there is a significant overlap between the transcriptional responses to nitrosative and oxidative stresses in *C. albicans*. As Hromatka and coworkers point out, this overlap is consistent with the observation that nitric oxide leads to the generation of reactive oxygen species, such as hydrogen peroxide, superoxide anions, and peroxynitrite. *C. albicans* genes encoding sulfur assimilation functions, transcription factors, and transporters are also induced in response to nitric oxide (84).

Weak Acid Stress Response

Weak acid stress occurs following exposure to compounds such as acetic, lactic, or benzoic acid, when the ambient pH is below their pK_a. Under these conditions, outside the cell the weak acid exists mainly in its relatively nonpolar form and can therefore diffuse across the membrane. Once it encounters the higher pH of the cytoplasm, the weak acid dissociates into its acid anionic form plus H^+. Given a high extracellular concentration of weak acid, the equilibrium drives this reaction towards uptake of the weak acid and the acidification of the cytoplasm. Yeast cells react by attempting to maintain the intracellular pH around neutrality by exporting protons in an energy-dependent fashion, and also by exporting the anion (143).

Following exposure to acetic acid, *C. albicans* responds by activating stress genes such as *HSP104*, *HSP90*, *CAP1*,

CTA4, and *MSN4* (149). Genes involved in Ras-cAMP signaling and glycogen metabolism are also induced. Interestingly, at higher doses of acetic acid, when programmed cell death is stimulated (139), *C. albicans* no longer mounts this adaptive response (149). The transcriptional response to weak acids is largely dependent upon the transcription factor Mnl1 (see below), and *C. albicans mnl1* cells are sensitive to a range of weak acids (149). This sensitivity is only seen at ambient pHs below the pK_a of the relevant weak acid. Hence, this represents sensitivity to weak acid stress rather than to the acid anion per se. The presumption is that for *C. albicans*, this Mnl1-regulated weak acid response is relevant during commensalism in the gastrointestinal tract, where the ambient pHs of some niches are low. However, this remains to be tested.

Heavy Metal Stress Response

The cellular levels of heavy metal ions in yeast are mainly controlled by sequestration with glutathione. Hence, heavy metals are thought to initiate an oxidative stress either through the depletion of cellular glutathione levels or by displacement of Zn^{2+} and Fe^{2+} ions from proteins, leading to hydroxyl radical production (76, 176). Therefore, one might expect overlap between the global transcriptional responses of *C. albicans* to heavy metals and oxidative stress. Some overlap has been observed (51), but of the 107 Cd^{2+}-induced genes and the 435 H_2O_2-induced genes, only 41 were induced by both stresses. Nevertheless, this gene set includes functions involved in oxidative stress resistance and glutathione biosynthesis: catalase (*CTA1*), a putative oxidoreductase (orf19.3131), a reductase (*GRP2*), dioxygenase (*IFH3*), gamma-glutamylcysteine synthetase (*GSH1*), and putative copper and zinc transporters (*CRD1* and *ZRT2*). This gene set also included the genes *CAP1* and *NRG1*. Cap1 plays a central role in the regulation of the response to oxidative stresses (see below), and Nrg1 has been shown to modulate stress responses in *C. albicans* (119, 120, 149, 198).

Some *C. albicans* genes are significantly induced by Cd^{2+} but not by H_2O_2 (51). These include genes involved in sulfate assimilation and the generation of sulfur-containing amino acids required for glutathione synthesis (such as *CYS3*, *MET1*, *MET2*, *MET3*, *MET10*, *MET12*, *MET14*, *MET15*, *MET16*, and *SUL1*), as well as glutathione biosynthesis itself (*GSH2*). This is consistent with the mechanistic link between heavy metal stress and glutathione homeostasis and with the global transcriptional response to Cd^{2+} observed in *S. cerevisiae* (57, 115, 194). In contrast, the primary response of *S. pombe* to Cd^{2+} appears to be the scavenging of environmental glutathione rather than the synthesis of new glutathione (38).

A second class of genes comprising HSP genes and chaperones is induced by *C. albicans* in response to Cd^{2+} (51). These genes contribute significantly to the general stress response in *S. cerevisiae* and *S. pombe*, but in *C. albicans* they appear to be induced specifically in response to Cd^{2+} rather than by oxidative or osmotic stresses. But how often are *C. albicans* cells exposed to a heavy metal stress in their commensal state or during infection?

General Stress Response

The above subsections focused on the specific cellular responses to particular stresses. However, following exposure to a range of diverse stresses such as heat, osmotic stress, or oxidative stresses, *S. cerevisiae* and *S. pombe* also activate large general stress responses. The same holds for *C. glabrata*, but *C. albicans* displays significant differences from these yeasts with respect to the general stress responses (51).

At first, the general stress response in *S. cerevisiae* was defined largely by the Msn2/4– stress-responsive element (Msn2/4-STRE) regulon, which activates a diverse range of cellular functions in response to a wide range of stresses (104). More recently, the general (or environmental) stress response has been defined as that set of genes that is commonly regulated in response to diverse stresses (such as heat shock, oxidative stress, pH stress, ethanol stress, and weak acid stress). General stress responses have been defined experimentally in *S. cerevisiae*, *S. pombe*, and *C. glabrata* (34, 38, 66, 153). There is considerable overlap between the Msn2/4-STRE regulon and general stress response genes in *S. cerevisiae*. However, not all general stress response genes in this yeast depend upon Msn2/4 for their activation: other transcription factors contribute to this response (34) (see below). Similarly, while Msn2 and Msn4 contribute significantly to the general stress response in *C. glabrata*, other regulators do contribute (153). In contrast, *S. pombe* regulates its general stress response by entirely different means—through the SAPK Sty1 and the Sty1-regulated transcription factor Atf1 (38).

General stress responses in budding and fission yeasts involve the regulation of several hundred genes that generally fall into four main classes (34, 38, 66). Firstly, many genes involved in DNA replication, transcription, and translation are downregulated, thereby reflecting the transient delay in growth while the yeast cells adapt to stress and repair damage. Secondly, genes involved in protecting against protein denaturation (HSPs or chaperones) are induced. Thirdly, genes involved in repairing oxidative damage and redox homeostasis (catalases, thioredoxins, and glutaredoxins) are induced. Fourthly, genes involved in carbohydrate metabolism are induced to generate the metabolic energy required for stress adaptation. The fourth class also includes genes involved in trehalose and glycogen metabolism. Trehalose acts as a stress protectant, accumulating in response to stresses and increasing the resistance of cells to these stresses (63, 133, 169, 183).

Initially it was thought that *C. albicans* does not display a general stress response, because no significant overlap was observed in the global transcriptional responses of *C. albicans* to mild heat (23 to 37°C), osmotic (0.3 M NaCl), and oxidative stresses (0.4 mM H_2O_2) (50). Also, no obvious stress cross-protection was observed when *C. albicans* cells were pretreated with a mild heat shock and then exposed to an oxidative stress (50). However, stress cross-protection is observed in *C. albicans* if cells are exposed to stresses that activate the SAPK Hog1 (172). Also, subsequent microarray experiments revealed a small subset of *C. albicans* genes that are commonly induced in response to stresses that activate Hog1 (0.3 M NaCl, 5 mM H_2O_2, and 0.5 mM $CdSO_4$ [51]). This *C. albicans* gene set was called the core stress response because it is significantly smaller than the gene sets representing the general stress response in budding and fission yeasts (51). (It should be noted that in model yeasts, the general stress response is sometimes called the core environmental stress response.) More recently, a core stress response was defined at the proteomic level in *C. albicans* (207). The cellular role of the core stress response in *C. albicans* is more specialized than the corresponding responses in *S. cerevisiae*, *S. pombe*, and *C. glabrata*, and a much narrower set of functional categories is enriched in the core stress genes in *C. albicans* than in these other yeasts (51, 153). Nevertheless, some classic stress genes were

identified in the core stress response in *C. albicans*, including catalase (*CTA1*), glycerol-3-phosphate dehydrogenase (*GPD2*), reductases (*GRP1*), trehalose-6-phosphate phosphatase (*TPS2*), a multidrug transporter (*CDR4*), and a Cu^{2+}-transporting ATPase (*CRD1*).

To summarize, the available data have revealed that *C. albicans* generally mounts *specialized* responses to *particular* stresses similar to those of the yeasts *S. cerevisiae*, *S. pombe*, and *C. glabrata*. For example, glycerol accumulation helps to protect *C. albicans* against hyperosmotic shock, trehalose protects *C. albicans* against a range of stresses, and glutathione and redox regulatory functions protect this pathogen against oxidative and nitrosative damage. However, there are clear differences between *C. albicans* and these other yeasts with respect to their core transcriptional responses to stress. Also, the pathogens *C. albicans* and *C. glabrata* are much more resistant to some stresses (particularly oxidative stress) than their benign cousins.

STRESS-SIGNALING PATHWAYS

Studies with the benign yeasts *S. cerevisiae* and *S. pombe* have provided significant insights into the molecular mechanisms that control stress responses in yeasts. While similar stress-signaling modules exist in *C. albicans*, it has become increasingly apparent that clear differences exist between the regulation of stress responses in these yeasts. Such differences probably reflect evolutionary adaptation to the contrasting environmental niches that these pathogenic and saprophytic yeasts occupy. This section describes our understanding of the molecular mechanisms that regulate stress responses in *C. albicans* and, where information is available, *C. glabrata*, contrasting these mechanisms mainly with those in *S. cerevisiae* and *S. pombe*.

MAPK Pathways

Mitogen-activated protein kinase (MAPK) pathways are important stress signaling modules found in all eukaryotes. Each comprises three sequential protein kinases. A MAPK kinase kinase (MAPKKK) phosphorylates and thereby activates a MAPKK, which subsequently phosphorylates a MAPK on conserved threonine and tyrosine residues located in the activation loop of the catalytic domain. This enhances the kinase activity and nuclear translocation of the MAPK, which results in the MAPK-dependent phosphorylation of various substrates (often transcription factors), thereby triggering an appropriate cellular response. In *C. albicans*, two MAPK modules are activated by stress: the Hog1 stress-activated MAPK and the p42-44 MAPK, Mkc1 (Fig. 1). The Cek1 and Cek2 MAPK modules contribute mainly to morphogenetic regulation, quorum sensing, and the mating response (39, 156, 199, 200) and are not discussed here.

The Hog1 Pathway

The *S. cerevisiae* Hog1 and the *S. pombe* Sty1 SAPK pathways are among the best-characterized stress-signaling systems in eukaryotes. The *S. pombe* Sty1 pathway is strongly activated in response to diverse stresses (185), coordinating a general stress response in this yeast (38). The primary function attributed to the Hog1 pathway in *S. cerevisiae* is to respond to osmotic stress, although several studies have subsequently implicated this SAPK in other stress responses (reviewed in reference 173). Similarly, *C. albicans* contains a single SAPK, called Hog1 (162). *C. albicans hog1* cells, like *S. cerevisiae hog1* cells, are sensitive to osmotic stress and fail to accumulate glycerol in response to osmotic stress (162) (Fig. 1). Subsequent studies have revealed that the Hog1 SAPK plays a broader role in *C. albicans*. Like Sty1 in *S. pombe*, *C. albicans* Hog1 is phosphorylated (and activated) in response to a wide range of environmental insults, including oxidative stress agents (6, 172), cell wall-damaging agents (118), heavy metal ions, the drug staurosporine, the purine analogue caffeine, the quorum-sensing molecule farnesol (172), increased glucose concentrations (152), and cationic peptides (195). Phosphorylation of Cek1 is enhanced when *hog1* cells are treated with the cell wall-stressing agents such as Ca^{2+} and calcofluor white, again pointing to cross talk between the Hog1 and other MAPK pathways in *C. albicans* (118). Significantly, *C. albicans hog1* cells display sensitivities to many of the stresses that activate this SAPK. Furthermore, Hog1 translocates to the nucleus following exposure to these stresses (12, 172) and Hog1 mediates osmotic stress-conferred cross-protection against high levels of oxidative stress (25 to 100 mM H_2O_2) in *C. albicans* (172). Mild temperature also appears to protect against subsequent exposure to high oxidative stress (50 mM H_2O_2), but since Hog1 is not activated immediately by such temperature upshifts, it is not surprising that this type of stress cross-protection is Hog1 independent (69). Nevertheless, it is clear that Hog1 signaling is crucial for the cellular responses of *C. albicans* to a wide range of stresses.

Similar to the SAPK modules in *S. cerevisiae* and *S. pombe*, Hog1 is regulated by a single MAPKK, Pbs2 (12). No Hog1 phosphorylation is detected in *C. albicans pbs2* cells in response to osmotic or oxidative stress, indicating that Pbs2 is the sole MAPKK that relays stress signals to the Hog1 SAPK. Furthermore, *pbs2* cells, like *hog1* cells, display sensitivity to osmotic and oxidative stresses, although interestingly, *pbs2* cells have been reported to be more sensitive than *hog1* cells to oxidative stress (12).

In *S. cerevisiae*, three MAPKKKs transduce signals to Pbs2: Ssk2, Ssk22, and Ste11 (103, 144). (Ste2 and Ste22 are paralogues that arose during the ancient whole-genome duplication event in the *S. cerevisiae* lineage.) *C. albicans* has orthologues of Ssk2/22 and Ste11, but the inactivation of Ste11 has no discernible impact upon Hog1 signaling. Instead, in *C. albicans*, Ssk2 is the sole MAPKKK that transduces osmotic and oxidative stress signals to Pbs2 and Hog1 (37). *C. glabrata* Hog1 is also activated in response to osmotic stress (73). However, *C. glabrata* ATCC 2001 (the main lineage that is used for molecular studies) carries an inactive, truncated *ssk2-1* allele (73). Consequently, *C. glabrata ste11* cells in this background are sensitive to osmotic stress (25), whereas *C. albicans ste11* cells are not (37). Most clinical isolates of *C. glabrata*, however, possess functional Ssk2 and Ste11 MAPKKKs.

Although the SAPK pathways in *S. cerevisiae*, *S. pombe*, and *C. albicans* respond to diverse stimuli, their activation profiles are not identical. For example, the *S. pombe* Sty1 and *S. cerevisiae* Hog1 SAPKs are rapidly activated following exposure to temperatures of 37°C or above. In contrast, Hog1 phosphorylation decreases immediately following a temperature upshift in *C. albicans* (69, 172). Furthermore, higher levels of oxidative stress are required to activate Hog1 in *C. albicans* than Sty1 in *S. pombe* (172). These differences no doubt reflect the evolutionary adaptation of these yeasts to their respective niches.

In *C. albicans*, the Hog1 SAPK pathway contributes to additional cellular processes. For example, Hog1 signaling has been implicated in morphogenetic regulation, as *hog1* and *pbs2* mutants display derepressed hyphal development

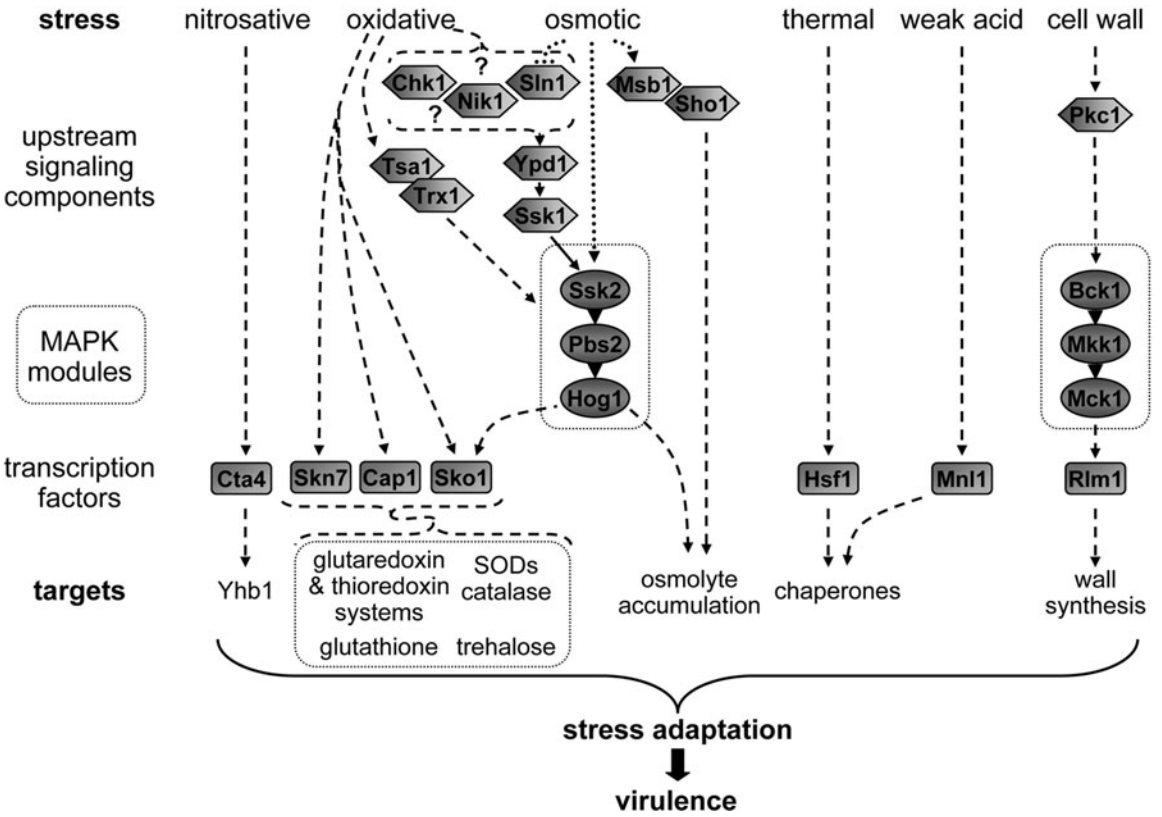

FIGURE 1 Diagram summarizing the pathways implicated in the adaptation of C. *albicans* to stress. See text for details. Most connections between signaling components are indicated by dashed lines, but dotted lines are used to indicate connections between osmotic stress signaling factors in an effort to distinguish them from oxidative stress signaling, particularly with regard to upstream components. 10.1128/9781555817176.ch15f1

in the absence of paramorphogens (5, 12, 48, 51). The data suggest that *HOG1* represses hyphal development in a manner that is independent of the cAMP-dependent protein kinase pathway and the Cek1 MAPK pathway (48). Hog1 is also required for chlamydospore formation (6). The inability of *hog1* cells to form chlamydospores is suppressed by the deletion of components of the Cek1 MAPK pathway. This suggests that it is the constitutive activation of Cek1 in *hog1* cells (154) that prevents chlamydospore formation (48). Furthermore, Hog1 and Pbs2 play roles in cell wall biogenesis, as mutants display altered growth characteristics compared to wild-type cells upon exposure to a range of compounds which disrupt the cell wall (5, 12). This phenotype is also linked to the hyperactivation of Cek1 in *hog1* cells (48). In addition, *hog1* cells display aberrant mitochondrial function (7), the basis of which is not clear. Finally, *HOG1* inactivation impairs the virulence of C. *albicans* in a mouse model of systemic candidiasis (5), and *hog1* cells are more susceptible to killing by macrophages or neutrophils (13). This emphasizes the importance of understanding the role of the Hog1 SAPK in regulating cellular responses in this pathogen.

Transcript profiling has been used to examine the role of Hog1 in C. *albicans* in osmotic, oxidative, and heavy metal stress responses as well as under basal (nonstressed) conditions (51). This confirmed that Hog1 plays a key role in the global transcriptional response to osmotic and heavy metal stress in C. *albicans*. For example, genes involved in sulfur amino acid biosynthesis, glycerol accumulation, and sugar and cation transport were deregulated in *hog1* cells. This was consistent with biochemical studies showing that Hog1 regulates stress-induced glycerol and polyol production in C. *albicans* (89, 162). However, the microarray data suggested that Hog1 does not play a major role in the transcriptional response to oxidative stress in C. *albicans*. This was surprising given that Hog1 is activated and accumulates in the nucleus in response to oxidative stress, and *HOG1* deletion confers oxidative stress sensitivity (6, 172). Such analyses might suggest that Hog1 contributes to the oxidative stress response at a posttranscriptional level in C. *albicans*. However, proteomic studies did not provide clear insights into the role of Hog1 in the oxidative stress response except to suggest that Hog1 might be required to ensure the prolonged expression of some proteins during recovery from such stresses (207). Furthermore, a recent report suggests that the tolerance of C. *albicans* to high doses of oxidative stress is independent of Hog1 (69). Nevertheless, it is clear that Hog1 plays an important, if as-yet-undefined, role in oxidative stress tolerance in C. *albicans* (6, 172).

Hog1 also regulates gene expression in the absence of stress (51, 207). Hypha-specific genes are significantly upregulated in *hog1* cells in the absence of stress, which

correlates with the observation that Hog1 acts as a repressor of morphogenesis (5, 12, 51). Other genes, many with stress-related functions, were also affected in *hog1* cells in the absence of stress, suggesting either that Hog1 is required for the basal level of expression of such genes or that *hog1* mutants are constitutively stressed. At the level of the proteome, inactivation of *HOG1* resulted in a significant remodeling of central carbon metabolism, as a number of glycolytic and tricarboxylic acid enzymes were downregulated, whereas certain pentose phosphate pathway enzymes were upregulated, in *hog1* cells (207).

While the components that comprise the Hog1 SAPK module have been identified, as have many downstream targets of this pathway, two key questions remain largely unanswered. How are stress signals relayed to the Hog1 module (see below)? Also, which cellular substrates are phosphorylated by Hog1 to mediate its effects upon stress responses, morphogenesis, and metabolism? Sko1 is one candidate, as this transcriptional repressor is phosphorylated in a Hog1-dependent manner (150) and regulates morphogenesis as well as contributing to the transcriptional response to oxidative stress in *C. albicans* (8).

The Mkc1 Pathway

The *C. albicans* Mkc1 MAPK pathway is orthologous to the Slt2/Mpk1 MAPK pathway in *S. cerevisiae* and is termed the cell integrity pathway (Fig. 1). Mkc1 is phosphorylated by the MAPKK Mkk2, which, in turn, is activated by the MAPKKK Bcy1 (155, 212). This MAPK cascade lies downstream of protein kinase C (Pkc1), which is not essential in *C. albicans* (138). *C. albicans* Mkc1 was first cloned via complementation of the temperature-sensitive defects of *S. cerevisiae slt2* mutants (124). *C. albicans* Mkc1 signaling, like Hog1, is also required for virulence (46). However, *C. albicans mkc1* cells are not especially sensitive to killing by macrophages or neutrophils (13). *C. albicans mkc1* cells are temperature sensitive and sensitive to cell wall-lytic enzymes and antifungals (124, 125, 196). Also, Mkc1 is phosphorylated in response to cell wall-damaging agents (118, 126), during filamentation and invasion on semisolid surfaces, and during biofilm formation (95). Mkc1 is also phosphorylated in a Hog1-dependent fashion in response to osmotic and oxidative stresses, although Mkc1 inactivation does not render cells sensitive to such stresses (12, 126). Therefore, Mkc1 signaling might not play a primary role in stress adaptation. Rather, this signaling pathway may play a more important role in the long-term maintenance of cell wall integrity in the face of genetic and environmental insults.

Two-Component Signal Transduction Pathways

Bacteria frequently exploit two-component signal transduction pathways to respond to environmental stimuli, and related pathways have been identified in fungi (36, 91, 163, 173). These pathways are generally more complex in eukaryotic cells: sequential His-Asp-His-Asp phosphorylation events are more common than the simple two-component pathways seen in prokaryotes. These multistep phosphorelay systems consist of a sensor histidine kinase, an intermediary phosphorelay protein, and a response regulator protein. In the benign yeasts *S. cerevisiae* and *S. pombe*, two-component signaling pathways sense and relay specific stress signals to the SAPK pathways (24, 127, 146). In *S. cerevisiae*, osmotic stress signals are transduced to Hog1 via two functionally redundant pathways: the Sln1 two-component signaling pathway and a separate module containing the transmembrane protein Sho1 (reviewed in reference 82). Sho1 was originally thought to function as the osmosensor at the top of this pathway (103). However, recent findings have implicated two transmembrane mucins, Msb2 and Hkr1, as the osmosensors in the Sho1 branch (182). In the Sln1 branch, in response to hyperosmotic stress the histidine kinase Sln1 becomes inactive, leading to the dephosphorylation of the response regulator Ssk1 (146). Ssk1 then interacts with the MAPKKK Ssk2 in the Hog1 pathway, and the dephosphorylated form of Ssk1 activates Ssk2 (145), which results in the activation of Hog1. A two-component system has also been identified in *S. pombe*, which, in contrast to *S. cerevisiae*, contains three histidine kinases, Mak1, Mak2, and Mak3 (24). Furthermore, this *S. pombe* pathway relays oxidative and not osmotic stress signals to the Sty1 SAPK (24, 127).

Several orthologues of two-component proteins have been identified in *C. albicans* (reviewed in reference 91). Three histidine kinases (Sln1, Chk1, and Nik1/COS) seem to represent composites of distinct histidine kinases found in three distantly related fungi (Fig. 1). *C. albicans* Sln1 is most similar to *S. cerevisiae* Sln1, the osmosensing histidine kinase in baker's yeast (122). Chk1 is a close homologue of Mak2 and Mak3 in *S. pombe*, which are oxidative stress sensors (30). Nik1 is most similar to the osmosensing Nik-1/COS kinase in *Neurospora crassa* (4, 122, 175). Like in *S. cerevisiae* and *S. pombe*, a single putative phosphorelay protein (Ypd1) has been identified in *C. albicans* (31). Furthermore, *C. albicans* expresses Ssk1, an orthologue of the *S. cerevisiae* response regulator Ssk1 (28). Significantly, two-component signal transduction is required for *C. albicans* pathogenicity, as mutants lacking two-component genes display decreased virulence in a murine model of systemic candidiasis (32, 33, 165, 205).

How are stress signals relayed to the Hog1 SAPK pathway? Deletion of the *C. albicans SSK1* gene prevents Hog1 activation in response to oxidative stress but not osmotic stress (35). Moreover, *C. albicans ssk1* cells are sensitive specifically to oxidative stress (35), which probably accounts for the observation that *ssk1* cells are more susceptible to neutrophil killing (47). Intriguingly, mutation of the conserved two-component Asp phosphorylation site of Ssk1 results in greater sensitivity to oxidative stress than that exhibited by *ssk1* cells. Moreover, although Hog1 is phosphorylated in response to peroxide stress in cells expressing this mutation, no nuclear accumulation of Hog1 is apparent (114). Collectively, these data suggest that in *C. albicans*, two-component signaling transduces oxidative stress signals, but not osmotic stress signals, to Hog1. However, based on the situation in *S. cerevisiae*, it was formally possible that the two-component pathway protein Ssk1 functions redundantly with the Sho1 pathway to relay osmotic stress signals to Hog1. But a *C. albicans ssk1 sho1* double mutant is not sensitive to osmotic stress, and the activation of Hog1 by osmotic stresses is not impaired in these cells (154). This contrasts with the situation in *S. cerevisiae* (103). Although these findings do not preclude a role for Ssk1 and Sho1 in osmotic stress signaling in *C. albicans*, they clearly indicate that an alternative osmotic signaling pathway exists in this pathogen. The mucin-like protein Msb2 contributes to osmoadaptation in *C. albicans*, as triple *ssk1 msb2 sho1* mutants are sensitive to osmotic stress (156). However, Msb2 does not appear to represent this alternative osmotic signaling pathway, as Hog1 is still activated in *ssk1 msb2 sho1* cells.

The response regulator Ssk1 is involved in the transmission of oxidative stress signals to Hog1, but which histidine

kinase acts as a peroxide sensor in C. *albicans*? The most likely candidate appears to be Chk1 because it shows significant sequence similarity to Mak2 and Mak3, the peroxide-sensing histidine kinases in *S. pombe* (24). C. *albicans chk1* cells do show oxidative stress sensitivity and are relatively sensitive to neutrophil killing (98, 187). Furthermore, C*HK1* gene expression is negatively regulated by Ssk1 and Hog1 (51, 98). However, inactivation of Chk1 does not block activation of Hog1 in response to oxidative stress (98). Furthermore, Hog1 is phosphorylated following exposure of *chk1 sln1* and *chk1 nik1* histidine kinase double mutants to oxidative stress (154). Hence, it is not clear how oxidative stress signals are relayed to the Ssk1 response regulator in C. *albicans*. Moreover, as Hog1 activation is seen in cells expressing Ssk1 lacking the predicted two-component regulated phosphoaspartate residue (114), it is possible that Ssk1 may regulate oxidative stress-induced phosphorylation of Hog1 via a mechanism independent of two-component signaling.

Two-component signal transduction has also been implicated in the regulation of morphogenesis, adherence, cell wall biosynthesis, and quorum sensing in C. *albicans* (4, 29, 32, 35, 92–94, 97, 205). It remains to be established exactly how two-component proteins sense and respond to the environmental cues that regulate such processes and whether this directly involves Hog1 signaling.

Redox-Sensitive Antioxidants as Regulators of Hog1

Redox-sensitive antioxidant proteins, with roles in the detoxification of reactive oxygen species, can also act as sensors and regulators of reactive oxygen species-induced signal transduction pathways. For example, oxidative stress-induced activation of the Sty1 SAPK also requires the thioredoxin peroxidase enzyme Tpx1, which acts downstream of the two-component pathway (192, 193). The precise mechanism of regulation of Sty1 by Tpx1 is unclear, but upon peroxide stress, intermolecular disulfide bonds are formed between conserved cysteine residues in Sty1 and Tpx1, suggestive of a direct role. In C. *albicans*, the analogous thioredoxin peroxidase enzyme, Tsa1, is also required specifically for the oxidative stress-induced activation of the Hog1 SAPK pathway (43). However, the mechanism of Tsa1 regulation of Hog1 in C. *albicans* may be different from that reported for *S. pombe*, as the peroxidatic catalytic cysteine residue, which is essential for Tpx1-mediated regulation of Sty1, is dispensable for Tsa1 regulation of Hog1 (43). Moreover, the thioredoxin enzyme Trx1, which regulates the redox status of Tsa1, is also essential for the relay of oxidative stress signals to the Hog1 SAPK module. Whether Trx1 and Tsa1 function in the same or distinct pathways to relay oxidative stress signals to Hog1 remains to be clarified.

TRANSCRIPTION FACTORS THAT DRIVE STRESS RESPONSES

Cap1

Cap1 is a key regulator of oxidative stress-induced gene transcription in C. *albicans*. Cap1 is a bZip transcription factor of the AP-1 family and is closely related to the *S. cerevisiae* Yap1 and *S. pombe* Pap1 proteins, which have well-studied roles in multidrug resistance and response to oxidative stress (41, 186). Cap1 was first identified through its ability to confer fluconazole resistance upon *S. cerevisiae* (1). In C. *albicans*, Cap1 inactivation causes sensitivity to a wide range of reactive oxygen species and heavy metals and to the drugs 4-nitroquinoline oxide and 1,10-phenanthroline (2, 6, 51, 211).

AP-1-like factors, including Cap1, activate the transcription of their target genes via the Yap response element (TKACTAA) (41, 58, 128). The significant enrichment of Yap response elements in the promoters of oxidative stress-induced genes suggested an important key role for Cap1 in oxidative stress gene transcription (51). This view was reinforced by Northern analyses and transcript profiling (51, 198) and by the definition of the Cap1 regulon by chromatin immunoprecipitation studies (214). For example, the largest groups of genes identified in this chromatin immunoprecipitation on analysis included those involved in oxidative stress resistance, such as *CTA1* and *TRX1*, and those involved in the response to drugs, such as *MDR1*. Notably, however, Cap1 binding was not restricted to promoters of these target genes, suggesting that Cap1 may remain bound to the transcriptional machinery or chromatin remodeling complexes during transcription (214). Indeed, a recent study revealed that an important function of Cap1 is to recruit the Ada2 component of the SAGA/ADA coactivator complex, which regulates histone acetylation, to the promoters of oxidative stress responsive target genes (166). This is clearly of functional significance, as *ada2* cells are highly sensitive to reactive oxygen species and the oxidative stress-induced transcription of Cap1 target genes such as *CTA1* and *TRX1* is significantly impaired (166). Although there are no data regarding the virulence of C. *albicans cap1* strains, it is likely that Cap1 will be necessary to survive host responses. For example, C*AP1* is strongly induced upon neutrophil exposure, and *cap1* cells display reduced viability when exposed to whole blood or neutrophils (62).

Like *S. cerevisiae* Yap1 and *S. pombe* Pap1, Cap1 accumulates in the C. *albicans* nucleus following exposure to H_2O_2 (211). In *S. cerevisiae*, Yap1 activation is mediated by changes in the redox status of two cysteine-rich domains (n-CRD and c-CRD) that prevent the interaction of Yap1 with the nuclear export factor Crm1 (44). Yap1 is not directly oxidized by H_2O_2; instead, Gpx3 (a glutathione peroxidase-like protein but which has thioredoxin peroxidase activity) catalyzes Yap1 disulfide bond formation. Conversely, activation of Yap1 is inhibited by the thioredoxins Trx1 and Trx2, which reduce both oxidized Gpx3 and Yap1 (45). Similarly, Cap1 contains n- and c-CRD regions, and mutation or truncation of the c-CRD affects Cap1 regulation (2, 211). Furthermore, Cap1 is oxidized following exposure of cells to H_2O_2, although in contrast to the case with *S. cerevisiae* Yap1, this is only partially inhibited upon inactivating the thioredoxin system in C. *albicans* (43). Hence, elements of the regulatory mechanisms identified in *S. cerevisiae* probably operate in C. *albicans* to some extent. However, C. *albicans* Cap1 and *S. pombe* Pap1 do display differences in their regulation. In *S. pombe* there is cross talk between the Sty1 SAPK and the Pap1 pathways (147, 185). For example, the nuclear accumulation of Pap1 depends upon Sty1, and Pap1 is inhibited at high peroxide concentrations. This is due to the fact that the thioredoxin peroxidase enzyme Tpx1, which mediates oxidation of Pap1, is inactivated at high peroxide concentrations. Moreover, Sty1 is required for induction of the sulfiredoxin-encoding *srx1*$^+$ gene, which restores the activity of the Tpx1 enzyme (18). However, in C. *albicans*, Cap1 rapidly accumulates in the nucleus in a Hog1-independent fashion at both low and

high H_2O_2 concentrations, and *SRX1* expression is Hog1 independent (6, 51). These findings are consistent with the idea that in *C. albicans*, Cap1 regulates the response to low and high levels of H_2O_2 independently of the Hog1 SAPK pathway.

Much less is known about the analogous transcription factor CgYap1 in *C. glabrata*, although cells lacking CgYap1 are sensitive to oxidative stress (42). Recent and impressive work by Roetzer et al. demonstrated that Yap1 in *C. glabrata* accumulates in the nucleus following phagocytosis by macrophages, in addition to H_2O_2 treatment in vitro (153).

Skn7

Orthologues of the *S. cerevisiae* Skn7 transcription factor have been identified and partially characterized in *C. albicans* (170) and *C. glabrata* (42, 160). The three main domains in *S. cerevisiae* Skn7 are conserved in the *Candida* proteins: a DNA-binding domain, a coiled-coil domain that probably mediates protein-protein interactions, and a receiver domain (analogous to those in response regulator proteins of two-component signal transduction pathways). *S. cerevisiae* Skn7 functions alongside Yap1 to regulate the activation of genes with antioxidant functions, and consequently the inactivation of *SKN7* renders cells sensitive to oxidative stress (96, 116). However, not much is known regarding the activation of Skn7, although it has been documented that Skn7 interacts directly with the heat shock transcription factor Hsf1 and mediates the induction of heat shock genes to oxidative stress (148). Furthermore, recent work has demonstrated that Skn7 is phosphorylated in response to oxidative stress in a Yap1-dependent manner (78). In this study, mutations in the Skn7 receiver domain were characterized that prevented both oxidative stress induced Skn7 phosphorylation and the formation of Skn7-Yap1 DNA complexes.

In both *C. albicans* and *C. glabrata*, cells lacking Skn7 display increased sensitivity to oxidants, suggesting that this protein functions to regulate oxidative stress-induced gene expression (42, 83, 160, 170). Furthermore, *C. glabrata* single or double *yap1 skn7* mutants are equally sensitive to H_2O_2 (42), suggesting that, as seen in *S. cerevisiae*, Skn7 functions alongside Yap1 in this fungal pathogen (Fig. 1). However, it is not known whether Skn7 acts alongside Cap1 in *C. albicans*. Both *C. albicans skn7* cells (170) and *C. glabrata skn7* cells (160) display only mildly attenuated virulence in mouse models of systemic candidiasis, indicating that the oxidative stress response mediated by Skn7 is not a major virulence attribute in these pathogenic fungi.

Msn4/Mnl1

As described above, *S. cerevisiae* activates a core stress response in response to diverse stresses such as mild heat shock, starvation, osmotic stress, alcohol, and weak acids. This transcriptional response is partially dependent upon Msn2 and Msn4 (34, 66, 164). Msn2 and Msn4 are closely related, partially functionally redundant $(C_2H_2)_2$ zinc finger transcription factors (54, 106). Msn2 and Msn4 are paralogues that arose as a result of the ancient whole-genome duplication event during *S. cerevisiae* evolution (203). There is a third member of this small family of transcription factors in *S. cerevisiae* (Yer130c), the function of which remains obscure.

In *S. cerevisiae*, Msn2 and Msn4 accumulate in the nucleus in response to a stress (70, 87), where they activate their target genes by binding to STREs in their promoters (CCCCT) (104, 108, 117). These transcription factors are downregulated by the Ras-cAMP pathway through protein kinase A-mediated phosphorylation of Msn2 and Msn4 (65, 70, 71), but their interaction with the STRE is enhanced by yeast glycogen synthase kinase 3 (80). It has recently been demonstrated that the two cytoplasmic thioredoxins Trx1 and Trx2 are essential for H_2O_2-stimulated nuclear accumulation of Msn2 (17). This is intriguing, as Trx1 and Trx2 function as inhibitors of Yap1 nuclear accumulation in *S. cerevisiae* by reducing oxidized active Yap1 (45). However, the precise molecular mechanisms underlying the regulation of Msn2 by thioredoxin remain unknown.

C. albicans has two Msn2/4-like proteins. Msn4 is orthologous to the paralogous *S. cerevisiae* pair, Msn2 and Msn4. Mnl1 is the orthologue of *S. cerevisiae* Yer130c (128, 149). Although many stress genes in *C. albicans* carry STRE-like elements, neither Msn4 nor Mnl1 plays a discernible role in controlling a core stress response in this pathogen (128). *C. albicans msn4 mnl1* double mutants do not display any sensitivities to thermal, osmotic, or oxidative stresses, and no significant differences were observed between the mutant and wild-type transcriptomes under these conditions (128). These observations are consistent with the observation that the core transcriptional response to stress in *C. albicans* has diverged significantly from that in *S. cerevisiae* (50, 51) and indicate that the Msn2/4-STRE regulon has been functionally reassigned in *C. albicans* compared with *S. cerevisiae*. Meanwhile, Msn2/4-STRE regulation has been retained in *C. glabrata*, as Msn2 and Msn4 contribute significantly to the core transcriptional response to stress in this pathogen (153). In this regard, it is noteworthy that the HD1 (homology domain 1) motif of Msn2/4, which is important for stress-regulated intracellular localization, is present only in close relatives of *S. cerevisiae* such as *C. glabrata* and not, for example, in *C. albicans* (153).

Interestingly, the Ras-cAMP pathway retains some control over the expression of osmotic stress genes in *C. albicans* (77), although Msn2/4 proteins are no longer involved in this response (128). Furthermore, *C. albicans* protein kinase A (*tpk1*) mutants are sensitive to salt, oxidative, and thermal stresses (67), and the high-affinity phosphodiesterase Pde2, which downregulates the Ras-cAMP pathway, is also required for stress resistance in *C. albicans* (202).

The other Msn2/4-like protein in *C. albicans*, Mnl1, plays a key role in the response to weak acids (149). Mnl1 activates the transcription of weak acid stress genes via a STRE-like element, and hence, the inactivation of Mnl1 compromises the long-term adaptation of *C. albicans* cells to weak acids.

Cta4

In response to nitrosative stress, *C. albicans* activates genes involved in the detoxification of reactive nitrogen species and the repair of damage caused by these toxins. These genes include those encoding nitric oxide dioxygenase, glutamate-cysteine ligase, glutathione *S*-transferase, glutathione reductase, glutathione *S*-conjugate transporter, glutaredoxin, thiol peroxidase, NADPH oxidoreductases, alternative oxidase, catalase, and flavohemoglobin (84). Not much is known about the transcriptional regulators that control this nitrosative stress response. However, the zinc finger transcription factor Cta4 has been identified as a key regulator of this response (40) (Fig. 1). At least some of these nitrosative stress genes are regulated by Cta4, and *C. albicans cta4* cells are sensitive to nitrosative stress. Cta4 was identified using

an elegant approach that first involved the elucidation of a nitric oxide-responsive element in the *YHB1* gene promoter (40). This regulatory DNA sequence was then used to isolate nitric oxide-responsive element binding proteins from *C. albicans* extracts, thereby leading to the identification of the transcription factor Cta4 as a major regulator of *YHB1* expression and resistance to nitrosative stress in *C. albicans* (40).

Hsf1

The levels of numerous transcripts, including *HSP104*, *HSP90*, and members of the *HSP70* family, are induced by heat shock in *C. albicans* (129). The regulation of these HSPs in *C. albicans* is similar to that in *S. cerevisiae*. In *S. cerevisiae*, heat shock elements (HSEs) in the promoters of *HSP* genes mediate their transcriptional activation in response to heat shock by the heat shock transcription factor Hsf1 (75, 206). In *S. cerevisiae*, Hsf1 is essential for viability (174, 201). Similarly, in *C. albicans*, *HSP* genes contain HSEs in their promoters (129, 161, 180), and these elements drive their transcriptional activation by Hsf1 in response to heat shock (129). *C. albicans* Hsf1 is activated by phosphorylation in response to heat shock, but it is also required for the basal expression of these essential chaperones, even in the absence of stress. Hence, *HSF1* is essential for viability in *C. albicans* (129). In *S. cerevisiae*, Hsf1 appears to play roles in additional stress responses, such as the oxidative stress response (74, 148). However, in *C. albicans*, the HSE-Hsf1 regulon appears to be specific for heat shock (129).

CONCLUSIONS AND PERSPECTIVES

In conclusion, it has become clear that the full virulence of *C. albicans* depends upon its ability to respond appropriately to environmental stresses. Clearly, during its evolution, *C. albicans* has retained orthologues of many of the stress genes present in *S. cerevisiae* and *S. pombe* (131). These include genes involved in stress detoxification (e.g., catalases and peroxidases), protection against the stresses (e.g., glutathione, glycerol, and trehalose biosynthetic enzymes), and damage repair functions (e.g., chaperonins and DNA repair enzymes). While these responses are mainly studied in vitro, they are relevant in vivo, representing important aspects of fungal responses to the host during the infection process. Significantly, *C. albicans* has become more resistant to osmotic and oxidative stresses than its benign cousins, *S. cerevisiae* and *S. pombe*. This is also the case for *C. glabrata*. This presumably reflects the relevance of these stress responses within host niches. How do specific adaptive mechanisms contribute to the physiological robustness of *C. albicans* and *C. glabrata* within different niches? This is a major question that remains to be addressed.

Compared with *C. albicans*, limited information is available about stress responses in other pathogenic *Candida* species. Nevertheless, clear differences are emerging among *C. albicans*, *C. glabrata*, and *C. dubliniensis*, for example. While *C. glabrata* is relatively resistant to osmotic and oxidative stress, *C. dubliniensis* is sensitive to these stresses as well as to heat shock. *C. glabrata* displays a core transcriptional response akin to that of *S. cerevisiae*, whereas *C. albicans* does not. The structures of Hog1 signaling networks differ between *C. glabrata* and *C. albicans*. As these differences must contribute to the behavior of these pathogens in their hosts, it is important that these differences are addressed at a molecular level. This relates to the above question about the physiological robustness of *Candida* cells in vivo. However, there is a bigger systems biology-related issue here. If we are to fully understand the nature of the interactions between these pathogenic *Candida* species and their human hosts, we must elucidate quantitatively the dynamic behaviors of these stress responses and their true contributions both spatially and temporally during disease establishment and progression. Quantitative mathematical modeling of these responses will provide an invaluable foil to our more classical molecular and genomic approaches.

Additionally, how does the rewiring of stress regulatory modules in *C. albicans* affect fungus-host interactions? As described above, it is apparent that during its evolution, *C. albicans* has retained many of the stress regulatory molecules that have been elucidated experimentally in *S. cerevisiae* and *S. pombe*. The Hog1 pathway, the Mkc1 cell integrity pathway, AP1-like transcription factors, the Hsf1 transcription factor, and Msn2/4-related transcription factors are all functional in *C. albicans*. However, the cellular roles of some of these regulatory modules have diverged in *C. albicans* with respect to *S. cerevisiae* and *S. pombe*. Clear examples of this include the significant differences in the different core transcriptional responses to stress in these yeasts and the functional specialization of Mnl1 in protecting *C. albicans* cells against weak acid stress. How does this rewiring affect colonization, commensalism, and infection?

A fourth major issue relates to the fact that in the wild, one particular stress is rarely imposed in isolation from other environmental insults. In other words, *Candida* cells are exposed simultaneously to combinations of stresses as they enter new microenvironments in vivo. In which case, how do *C. albicans* or *C. glabrata* cells respond to such combinatorial stresses in these niches, for example, to oxidative plus nitrosative stress or to oxidative plus osmotic stress? Do these stresses have an impact additively or synergistically upon the *Candida* cells?

Finally, how is stress regulation integrated with growth, morphogenesis, and cell death? It was predicted many years ago that stress responses might be intimately connected with morphogenesis in *C. albicans* (23). While the predicted connections have subsequently been disproven, the principle clearly stands that stress adaptation, growth, and morphogenesis are intimately linked. For example, temperature upshifts and oxidative stress stimulate morphogenesis, while osmotic stress represses morphogenesis (43, 102, 123). Hog1 represses hyphal development (5). Regulators of oxidative stress adaptation also control morphogenesis (8, 32, 43). Furthermore, exposure to glucose can stimulate morphogenesis and also enhances resistance to oxidative stress (85, 105, 152). Also, both Ras-cAMP signaling and the suppression of normal transcriptional responses to stress have been associated with programmed cell death in *C. albicans* (140, 149). Further investigation of the molecular links among stress adaptation, growth, morphogenesis, and cell death is clearly warranted.

We gratefully acknowledge our friends and colleagues for many stimulating discussions on fungal stress responses and pathogenicity. We also thank our funding bodies for their support. A.J.P.B. and N.A.R.G. are funded by the UK Biotechnology and Biological Sciences Research Council (BB/F00513X/1, BB/D009308/1, BB/F000111/1, and BB/F010826/1), the Wellcome Trust (080088 and 086827), and the European Commission (PITN-GA-2008-214004, ERC-2009-AdG-249793, SIGNALPATH, and ARIADNE). J.Q. is funded by the Wellcome Trust (086048 and 089930).

REFERENCES

1. **Alarco, A. M., I. Balan, D. Talibi, N. Mainville, and M. Raymond.** 1997. AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. *J. Biol. Chem.* **272:**19304–19313.
2. **Alarco, A. M., and M. Raymond.** 1999. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J. Bacteriol.* **181:**700–708.
3. **Albertyn, J., S. Hohmann, J. M. Thevelein, and B. A. Prior.** 1994. *GPD1*, which encodes glycerol-3-phosphate dehydrogenase, is essential for growth under osmotic stress in *Saccharomyces cerevisiae*, and its expression is regulated by the element. *EMBO J.* **10:**585–592.
4. **Alex, L. A., C. Korch, C. P. Selitrennikoff, and M. I. Simon.** 1998. *COS1*, a two-component histidine kinase that is involved in hyphal development in the opportunistic pathogen *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **95:**7069–7073.
5. **Alonso-Monge, R., F. Navarro-Garcia, G. Molero, R. Diez-Orejas, M. Gustin, J. Pla, M. Sanchez, and C. Nombela.** 1999. Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J. Bacteriol.* **181:**3058–3068.
6. **Alonso-Monge, R., F. Navarro-Garcia, E. Roman, A. I. Negredo, B. Eisman, C. Nombela, and J. Pla.** 2003. The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*. *Eukaryot. Cell* **2:**351–361.
7. **Alonso-Monge, R., S. Carvaihlo, C. Nombela, E. Rial, and J. Pla.** 2009. The Hog1 MAP kinase controls respiratory metabolism in the fungal pathogen *Candida albicans*. *Microbiology* **155:**413–423.
8. **Alonso-Monge, R., E. Roman, D. M. Arana, D. Prieto, V. Urrialde, C. Nombela, and J. Pla.** 2010. The Sko1 protein represses the yeast-to-hypha transition and regulates the oxidative stress response in *Candida albicans*. *Fungal Genet. Biol.* **47:**587–601.
9. **Alvarez-Peral, F. J., O. Zaragoza, Y. Pedreno, and J. C. Arguelles.** 2002. Protective role of trehalose during severe oxidative stress caused by hydrogen peroxide and the adaptive oxidative stress response in *Candida albicans*. *Microbiology* **148:**2599–2606.
10. **Alves, S. H., E. P. Milan, P. de Laet Santana, L. O. Oliveira, J. M. Santurio, and A. L. Colombo.** 2002. Hypertonic Sabouraud broth as a simple and powerful test for *Candida dubliniensis* screening. *Diagn. Microbiol. Infect. Dis.* **43:**85–86.
11. **Ansell, R., K. Granath, S. Hohmann, J. M. Thevelein, and L. Adler.** 1997. The two isoenzymes for yeast NAD-dependent glycerol 3-phosphate dehydrogenase encoded by *GPD1* and *GPD2* have distinct roles in osmoadaptation and redox regulation. *EMBO J.* **16:**2179–2187.
12. **Arana, D. M., C. Nombela, R. Alonso-Monge, and J. Pla.** 2005. The Pbs2 MAP kinase kinase is essential for the oxidative-stress response in the fungal pathogen *Candida albicans*. *Microbiology* **151:**1033–1049.
13. **Arana, D. M., R. Alonso-Monge, C. Du, R. A. Calderone, and J. Pla.** 2007. Differential susceptibility of mitogen-activated protein kinase pathway mutants to oxidative-mediated killing by phagocytes in the fungal pathogen *Candida albicans*. *Cell. Microbiol.* **9:**1647–1659.
14. **Arguelles, J. C.** 1997. Thermotolerance and trehalose accumulation induced by heat shock in yeast cells of *Candida albicans*. *FEMS Microbiol. Lett.* **146:**65–71.
15. **Bambach, A., M. P. Fernandes, A. Ghosh, M. Kruppa, D. Alex, D. Li, W. A. Fonzi, N. Chauhan, N. Sun, O. A. Agrellos, A. E. Vercesi, R. J. Rolfes, and R. A. Calderone.** 2009. Goa1p of *Candida albicans* localizes to the mitochondria during stress and is required for mitochondrial function and virulence. *Eukaryot. Cell* **8:**1706–1720.
16. **Blasi, E., L. Pitzurra, M. Puliti, A. R. Chimenti, R. Mazzolla, R. Barluzzi, and F. Bistoni.** 1995. Differential susceptibility of yeast and hyphal forms of *Candida albicans* to macrophage-derived nitrogen-containing compounds. *Infect. Immun.* **63:**1806–1809.
17. **Boisnard, S., G. Lagniel, C. Garmendia-Torres, M. Molin, E. Boy-Marcotte, M. Jacquet, M. B. Toledano, J. Labarre, and S. Chedin.** 2009. H_2O_2 activates the nuclear localization of Msn2 and Maf1 through thioredoxins in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **8:**1429–1438.
18. **Bozonet, S. M., V. J. Findlay, A. M. Day, J. Cameron, E. A. Veal, and B. A. Morgan.** 2005. Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide. *J. Biol. Chem.* **280:**23319–23327.
19. **Brock, M.** 2009. Fungal metabolism in host niches. *Curr. Opin. Microbiol.* **12:**371–376.
20. **Bromuro, C., R. la Valle, S. Sandini, F. Urbani, C. M. Ausiello, L. Morelli, C. F. d'Ostiani, L. Romani, and A. Cassone.** 1998. A 70-kilodalton recombinant heat shock protein of *Candida albicans* is highly immunogenic and enhances systemic murine candidiasis. *Infect. Immun.* **66:**2154–2162.
21. **Brown, A. J. P.** 2005. Integration of metabolism with virulence in *Candida albicans*, p. 185–203. *In* A. J. P. Brown (ed.), *Mycota*, vol. XIII. *Fungal Genomics*. Springer-Verlag, Heidelberg, Germany.
22. **Brown, A. J. P., and N. A. R. Gow.** 1999. Regulatory networks controlling *Candida albicans* morphogenesis. *Trends Microbiol.* **7:**333–338.
23. **Brown, A. J. P., C. J. Barelle, S. Budge, J. Duncan, S. Harris, P. R. Lee, P. Leng, S. Macaskill, A. M. Abdul Murad, M. Ramsdale, C. Wiltshire, J. A. Wishart, and N. A. R. Gow.** 2000. Gene regulation during morphogenesis in *Candida albicans*, p. 112–125. *In* J. F. Ernst and A. Schmidt (ed.), *Contributions to Microbiology*, vol. 5. *Dimorphism in Human Pathogenic and Apathogenic Yeasts*. Karger AG, Basel, Switzerland.
24. **Buck, V., J. Quinn, T. Soto Pino, H. Martin, J. Saldanha, K. Makino, B. A. Morgan, and J. B. Millar.** 2001. Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. *Mol. Biol. Cell* **12:** 407–419.
25. **Calcagno, A. M., E. Bignell, T. R. Rogers, M. D. Jones, F. A. Muhlschlegel, and K. Haynes.** 2005. *Candida glabrata* Ste11 is involved in adaptation to hypertonic stress, maintenance of wild-type levels of filamentation and plays a role in virulence. *Med. Mycol.* **43:**355–364.
26. **Calderone, R. A.** 2002. Candida *and Candidiasis*. ASM Press, Washington, DC.
27. **Calderone, R. A., and W. A. Fonzi.** 2001. Virulence factors of *Candida albicans*. *Trends Microbiol.* **9:**327–335.
28. **Calera, J. A., and R. A. Calderone.** 1999. Identification of a putative response regulator two-component phosphorelay gene (*CaSSK1*) from *Candida albicans*. *Yeast* **15:** 1243–1254.
29. **Calera, J. A., and R. A. Calderone.** 1999. Flocculation of hyphae is associated with a deletion in the putative CaHK1 two-component histidine kinase gene from *Candida albicans*. *Microbiology* **145:**1431–1442.
30. **Calera, J. A., G. H. Choi, and R. A. Calderone.** 1998. Identification of a putative histidine kinase two-component phosphorelay gene (*CaHK1*) in *Candida albicans*. *Yeast* **14:**665–674.
31. **Calera, J. A., D. Herman, and R. A. Calderone.** 2000. Identification of *YPD1*, a gene of *Candida albicans* which

encodes a two-component phosphohistidine intermediate protein. *Yeast* **16:**1053–1059.
32. **Calera, J. A., X. J. Zhao, and R. A. Calderone.** 2000. Defective hyphal development and avirulence caused by a deletion of the *SSK1* response regulator gene in *Candida albicans*. *Infect. Immun.* **68:**518–525.
33. **Calera, J. A., X. J. Zhao, F. De Bernardis, M. Sheridan, and R. A. Calderone.** 1999. Avirulence of *Candida albicans* CaHK1 mutants in a murine model of hematogenously disseminated candidiasis. *Infect. Immun.* **67:** 4280–4284.
34. **Causton, H. C., B. Ren, S. S. Koh, C. T. Harbison, E. Kanin, E. G. Jennings, T. I. Lee, H. L. True, E. S. Lander, and R. A. Young.** 2001. Remodeling of yeast genome expression in response to environmental changes. *Mol. Biol. Cell* **12:**323–337.
35. **Chauhan, N., D. Inglis, E. Roman, J. Pla, D. Li, J. A. Calera, and R. A. Calderone.** 2003. *Candida albicans* response regulator gene *SSK1* regulates a subset of genes whose functions are associated with cell wall biosynthesis and adaptation to oxidative stress. *Eukaryot. Cell* **2:** 1018–1024.
36. **Chauhan, N., J. P. Latge, and R. A. Calderone.** 2006. Signalling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. *Nat. Rev. Microbiol.* **4:**435–444.
37. **Cheetham, J., D. A. Smith, A. da Silva Dantas, K. S. Doris, M. J. Patterson, C. R. Bruce, and J. Quinn.** 2007. A single MAPKKK regulates the Hog1 MAPK pathway in the pathogenic fungus *Candida albicans*. *Mol. Biol. Cell* **18:**4603–4614.
38. **Chen, D., W. M. Toone, J. Mata, R. Lyne, G. Burns, K. Kivinen, A. Brazma, N. Jones, and J. Bahler.** 2003. Global transcriptional responses of fission yeast to environmental stress. *Mol. Biol. Cell* **14:**214–229.
39. **Chen, J., J. Chen, S. Lane, and H. Liu.** 2002. A conserved mitogen activated protein kinase pathway is required for mating in *Candida albicans*. *Mol. Microbiol.* **46:**1335–1344.
40. **Chiranand, W., I. McLeod, H. Zhou, J. L. Lynn, L. A. Vega, H. Myers, J. R. Yates III, M. C. Lorenz, and M. C. Gustin.** 2008. *CTA4* transcription factor mediates induction of nitrosative stress response in *Candida albicans*. *Eukaryot. Cell* **7:**268–278.
41. **Cohen, B. A., Y. Pilpel, R. D. Mitra, and G. M. Church.** 2002. Discrimination between paralogs using microarray analysis: application to the Yap1 and Yap2 transcriptional networks. *Mol. Biol. Cell* **13:**1608–1614.
42. **Cuellar-Cruz, M., M. Briones-Martin-del-Campo, I. Canas-Villamar, J. Montalvo-Arredondo, L. Riego-Ruiz, I. Castano, and A. De Las Penas.** 2008. High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. *Eukaryot. Cell* **7:**814–825.
43. **da Silva Dantas, A., M. J. Patterson, D. A. Smith, D. M. MacCallum, L. P. Erwig, B. A. Morgan, and J. Quinn.** 2010. Thioredoxin regulates multiple hydrogen peroxide-induced signaling pathways in *Candida albicans*. *Mol. Cell. Biol.* **30:**4550–4563.
44. **Delaunay, A., A. D. Isnard, and M. B. Toledano.** 2000. H_2O_2 sensing through oxidation of the Yap1 transcription factor. *EMBO J.* **19:**5157–5166.
45. **Delaunay, A., D. Pflieger, M. B. Barrault, J. Vinh, and M. B. Toledano.** 2002. A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. *Cell* **111:**471–481.
46. **Diez-Orejas, R., G. Molero, F. Navarro-Garcia, J. Pla, C. Nombela, and M. Sanchez-Perez.** 1997. Reduced virulence of *Candida albicans MKC1* mutants: a role for mitogen-activated protein kinase in pathogenesis. *Infect. Immun.* **65:**833–837.
47. **Du, C., R. A. Calderone, J. Richert, and D. Li.** 2005. Deletion of the *SSK1* response regulator gene in *Candida albicans* contributes to enhanced killing by human polymorphonuclear neutrophils. *Infect. Immun.* **73:**865–871.
48. **Eisman, B., R. Alonso-Monge, E. Roman, D. Arana, C. Nombela, and J. Pla.** 2006. The Cek1 and Hog1 mitogen-activated protein kinases play complementary roles in cell wall biogenesis and chlamydospore formation in the fungal pathogen *Candida albicans*. *Eukaryot. Cell* **5:**347–358.
49. **Elahi, S., G. Pang, R. B. Ashman, and R. Clancy.** 2001. Nitric oxide-enhanced resistance to oral candidiasis. *Immunology* **104:**447–454.
50. **Enjalbert, B., A. Nantel, and M. Whiteway.** 2003. Stress-induced gene expression in *Candida albicans*: absence of a general stress response. *Mol. Biol. Cell* **14:**1460–1467.
51. **Enjalbert, B., D. A. Smith, M. J. Cornell, I. Alam, S. Nicholls, A. J. P. Brown, and J. Quinn.** 2006. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. *Mol. Biol. Cell* **17:**1018–1032.
52. **Enjalbert, B., D. MacCallum, F. C. Odds, and A. J. P. Brown.** 2007. Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. *Infect. Immun.* **75:**2143–2151.
53. **Enjalbert, B., G. P. Moran, C. Vaughan, T. Yeomans, D. M. MacCallum, J. Quinn, D. C. Coleman, A. J. P. Brown, and D. J. Sullivan.** 2009. Genome-wide gene expression profiling and forward genetic screens show that differential expression of the sodium ion transporter Ena21 contributes to the differential tolerance of *Candida albicans* and *Candida dubliniensis* to osmotic stress. *Mol. Microbiol.* **72:**216–228.
54. **Estruch, F., and M. Carlson.** 1993. Two homologous zinc finger genes identified by multicopy suppression in a SNF1 protein kinase mutant of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **13:**3872–3881.
55. **Fan, J. J., M. Whiteway, and S. H. Shen.** 2005. Disruption of a gene encoding glycerol 3-phosphatase from *Candida albicans* impairs intracellular glycerol accumulation-mediated salt-tolerance. *FEMS Microbiol. Lett.* **245:** 107–116.
56. **Fang, F. C.** 2004. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies. *Nat. Rev. Microbiol.* **2:**820–832.
57. **Fauchon, M., G. Lagniel, J. C. Aude, L. Lombardia, P. Soularue, C. Petat, G. Marguerie, A. Sentenac, M. Werner, and J. Labarre.** 2002. Sulfur sparing in the yeast proteome in response to sulfur demand. *Mol. Cell* **9:**713–723.
58. **Fernandes, L., C. Rodrigues-Pousada, and K. Struhl.** 1997. Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. *Mol. Cell. Biol.* **17:**6982–6993.
59. **Fidel, P. L., Jr.** 2004. History and new insights into host defense against vaginal candidiasis. *Trends Microbiol.* **12:** 220–227.
60. **Fidel, P. L., Jr., M. M. Barousse, T. Espinosa, M. Ficarra, J. Sturtevant, D. H. Martin, A. J. Quayle, and K. Dunlap.** 2004. A live intravaginal *Candida* challenge in humans reveals new hypothesis for the immunopathogenesis of vulvovaginal candidiasis. *Infect. Immun.* **72:**2939–2946.
61. **Fradin, C., M. Kretschmar, T. Nichterlein, C. Gaillardin, C. d'Enfert, and B. Hube.** 2003. Stage-specific gene expression of *Candida albicans* in human blood. *Mol. Microbiol.* **47:**1523–1543.
62. **Fradin, C., P. De Groot, D. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube.** 2005. Granulocytes govern the transcriptional response, morphology and proliferation

of *Candida albicans* in human blood. *Mol. Microbiol.* **56:**397–415.
63. **Francois, J., and J. L. Parrou.** 2001. Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **25:**125–145.
64. **Frohner, I. E., C. Bourgeois, K. Yatsyk, O. Majer, and K. Kuchler.** 2009. *Candida albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance *Mol. Microbiol.* **71:** 240–252.
65. **Garreau, H., R. N. Hasa, G. Renault, F. Estruch, E. Boy-Marcotte, and M. Jacquet.** 2000. Hyperphosphorylation of Msn2 and Msn4 in response to heat shock and the diauxic shift is inhibited by cAMP in *Saccharomyces cerevisiae*. *Microbiology* **146:**2113–2120.
66. **Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown.** 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11:**4241–4257.
67. **Giacometti, R., F. Kronberg, R. M. Biondi, and S. Passeron.** 2009. Catalytic isoforms Tpk1 and Tpk2 of *Candida albicans* PKA have non-redundant roles in stress response and glycogen storage. *Yeast* **26:**273–285.
68. **Gonzalez-Parraga, P., J. A. Hernandez, and J. C. Arguelles.** 2003. Role of antioxidant enzymatic defences against oxidative stress H_2O_2 and the acquisition of oxidative tolerance in *Candida albicans*. *Yeast* **20:**1161–1169.
69. **Gonzalez-Parraga, P., R. Alonso-Monge, J. Pla, and J. C. Arguelles.** 2010. Adaptive tolerance to oxidative stress and the induction of antioxidant enzymatic activities in *Candida albicans* are independent of the Hog1 and Cap1-mediated pathways. *FEMS Yeast Res.* doi:10.1111/j.1567-1364.2010.00654.x.
70. **Gorner, W., E. Durchschlag, M. T. Martinez-Pastor, F. Estruch, G. Ammerer, B. Hamilton, H. Ruis, and C. Schuller.** 1998. Nuclear localisation of the C_2H_2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev.* **12:**586–597.
71. **Gorner, W., E. Durchschlag, J. Wolf, E. L. Brown, G. Ammerer, B. Hamilton, H. Ruis, and C. Schuller.** 2002. Acute glucose starvation activates the nuclear localization signal of a stress-specific yeast transcription factor. *EMBO J.* **21:**135–144.
72. **Grant, C. M.** 2001. Role of the glutathione/glutaredoxin and thioredoxin systems in yeast growth and response to stress. *Mol. Microbiol.* **39:**533–541.
73. **Gregori, C., C. Schuller, A. Roetzer, T. Schwarzmuller, G. Ammerer, and K. Kuchler.** 2007. The high-osmolarity glycerol response pathway in the human fungal pathogen *Candida glabrata* strain ATCC 2001 lacks a signaling branch that operates in baker's yeast. *Eukaryot. Cell* **6:**1635–1645.
74. **Hahn, J. S., and D. J. Thiele.** 2004. Activation of the *Saccharomyces cerevisiae* heat shock transcription factor under glucose starvation conditions by Snf1 protein kinase. *J. Biol. Chem.* **279:**5169–5176.
75. **Hahn, J. S., Z. Hu, D. J. Thiele, and V. R. Iyer.** 2004. Genome-wide analysis of the biology of stress responses through heat shock transcription factor. *Mol. Cell. Biol.* **24:**5249–5256.
76. **Halliwell, B., and J. M. Gutteridge.** 1984. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem. J.* **219:**1–14.
77. **Harcus, D., A. Nantel, A. Marcil, T. Rigby, and M. Whiteway.** 2004. Transcription profiling of cyclic AMP signaling in *Candida albicans*. *Mol. Biol. Cell* **15:**4490–4499.
78. **He, X. J., K. E. Mulford, and J. S. Fassler.** 2009. Oxidative stress function of the *Saccharomyces cerevisiae* Skn7 receiver domain. *Eukaryot. Cell* **8:**768–778.
79. **Hersen, P., M. N. McClean, L. Mahadevan, and S. Ramanathan.** 2008. Signal processing by the HOG MAP kinase pathway. *Proc. Natl. Acad. Sci USA* **105:** 7165–7170.
80. **Hirata, Y., T. Andoh, T. Asahara, and A. Kikuchi.** 2003. Yeast glycogen synthase kinase-3 activates Msn2p-dependent transcription of stress responsive genes. *Mol. Biol. Cell* **14:**302–312.
81. **Hirayama, T., T. Maeda, H. Saito, and K. Shinozaki.** 1995. Cloning and characterization of seven cDNAs for hyperosmolarity-responsive (*HOR*) genes of *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **249:**127–138.
82. **Hohmann, S.** 2002. Osmotic stress signaling and osmoadaptation in yeasts. *Microbiol. Mol. Biol. Rev.* **66:** 300–372.
83. **Homann, O. R., J. Dea, S. M. Noble, and A. D. Johnson.** 2009. A phenotypic profile of the *Candida albicans* regulatory network. *PLoS Genet.* **5:**e1000783.
84. **Hromatka, B. S., S. M. Noble, and A. D. Johnson.** 2005. Transcriptional response of *Candida albicans* to nitric oxide and the role of the *YHB1* gene in nitrosative stress and virulence. *Mol. Biol. Cell* **16:**4814–4826.
85. **Hudson, D. A., Q. L. Sciascia, R. J. Sanders, G. E. Norris, P. J. B. Edwards, P. A. Sullivan, and P. C. Farley.** 2004. Identification of the dialysable serum inducer of germ-tube formation in *Candida albicans*. *Microbiology* **150:**3041–3049.
86. **Hwang, C. S., G. E. Rhie, J. H. Oh, W. K. Huh, H. S. Yim, and S. O. Kang.** 2002. Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. *Microbiology* **148:**3705–3713.
87. **Jacquet, M., G. Renault, S. Lallet, J. De Mey, and A. Goldbeter.** 2003. Oscillatory nucleocytoplasmic shuttling of the general stress response transcriptional activators Msn2 and Msn4 in *Saccharomyces cerevisiae*. *J. Cell Biol.* **161:**497–505.
88. **Jamieson, D. J., D. W. Stephen, and E. C. Terriere.** 1996. Analysis of the adaptive oxidative stress response of *Candida albicans*. *FEMS Microbiol. Lett.* **138:**83–88.
89. **Kayingo, G., and B Wong.** 2005. The MAP kinase Hog1p differentially regulates stress-induced production and accumulation of glycerol and D-arabitol in *Candida albicans*. *Microbiology* **151:**2987–2999.
90. **Klipp, E., B. Nordlander, R. Kruger, P. Gennemark, and S. Hohmann.** 2005. Integrative model of the response of yeast to osmotic shock. *Nat. Biotechnol.* **23:**975–982.
91. **Kruppa, M., and R. A. Calderone.** 2006. Two-component signal transduction in human fungal pathogens. *FEMS Yeast Res.* **6:**149–159.
92. **Kruppa, M., T. Goins, J. E. Cutler, D. Lowman, D. Williams, N. Chauhan, V. Menon, P. Singh, D. Li, and R. A. Calderone.** 2003. The role of the *Candida albicans* histidine kinase (*CHK1*) gene in the regulation of cell wall mannan and glucan biosynthesis. *FEMS Yeast Res.* **3:**289–299.
93. **Kruppa, M., M. A. Jabra-Rizk, T. F. Meiller, and R. A. Calderone.** 2004. The histidine kinases of *Candida albicans*: regulation of cell wall mannan biosynthesis. *FEMS Yeast Res.* **4:**409–416.
94. **Kruppa, M., B. P. Krom, N. Chauhan, A. V. Bambach, R. L. Cihlar, and R. A. Calderone.** 2004. The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. *Eukaryot. Cell* **3:** 1062–1065.
95. **Kumamoto, C. A.** 2005. A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. *Proc. Natl. Acad. Sci. USA* **102:**5576–5581.

96. **Lee, J., C. Godon, G. Lagniel, D. Spector, J. Garin, J. Labarre, and M. B. Toledano.** 1999. Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. *J. Biol. Chem.* **274:**16040–16046.
97. **Li, D., J. Bernhardt, and R. A. Calderone.** 2002. Temporal expression of the *Candida albicans* genes *CHK1* and *CSSK1*, adherence, and morphogenesis in a model of reconstituted human esophageal epithelial candidiasis. *Infect. Immun.* **70:**1558–1565.
98. **Li, D., V. Gurkovska, M. Sheridan, R. A. Calderone, and N. Chauhan.** 2004. Studies on the regulation of the two-component histidine kinase gene *CHK1* in *Candida albicans* using the heterologous lacZ reporter gene. *Microbiology* **150:**3305–3313.
99. **Lorenz, M. C., and G. R. Fink.** 2001. The glyoxylate cycle is required for fungal virulence. *Nature* **412:**83–86.
100. **Lorenz, M. C., J. A. Bender, and G. R. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot. Cell* **3:**1076–1087.
101. **Luyten, K., J. Albertyn, W. F. Skibbe, B. A. Prior, J. Ramos, J. M. Thevelein, and S. Hohmann.** 1995. Fps1, a yeast member of the MIP family of channel proteins, is a facilitator for glycerol uptake and efflux and is inactive under osmotic stress. *EMBO J.* **14:**1360–1371.
102. **Macaskill, S.** 2003. Functional analysis of specific promoter elements involved in the control of *Candida albicans* transcription. Ph.D. thesis. University of Aberdeen, Aberdeen, United Kingdom.
103. **Maeda, T., M. Takekawa, and H. Saito.** 1995. Activation of yeast PBS2 MAPKK by MAPKKKs or by binding of an SH3-containing osmosensor. *Science* **269:**554–558.
104. **Mager, W. H., and A. J. J. de Kruijff.** 1995. Stress-induced transcriptional activation. *Microbiol. Rev.* **59:** 506–531.
105. **Maidan, M. M., J. M. Thevelein, and P. Van Dijck.** 2005. Carbon source induced yeast-to-hypha transition in *Candida albicans* is dependent on the presence of amino acids and on the G-protein-coupled receptor Gpr1. *Biochem. Soc. Trans.* **33:**291–293.
106. **Marchler, G., C. Schuller, G. Adam, and H. Ruis.** 1993. A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. *EMBO J.* **12:**1997–2003.
107. **Martchenko, M., A. M. Alarco, D. Harcus, and M. Whiteway.** 2004. Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. *Mol. Biol. Cell* **15:**456–467.
108. **Martinez-Pastor, M. T., G. Marchler, C. Schuller, A. Marchler-Bauer, H. Ruis, and F. Estruch.** 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress-response element. *EMBO J.* **15:**2227–2235.
109. **Matthews, R. C.** 1992. *Candida albicans HSP90*: link between protection and autoimmunity. *J. Med. Microbiol.* **36:**367–370.
110. **Matthews, R. C., J. P. Burnie, and S. Tabaqchali.** 1987. Isolation of immunodominant antigens from the sera of patients with systemic candidiasis and characterisation of the serological response to *Candida albicans*. *J. Clin. Microbiol.* **25:**230–237.
111. **Matthews, R. C., J. P. Burnie, D. Smith, I. Clark, J. Midgley, M. Conolly, and B. Gazzard.** 1988. *Candida* and AIDS: evidence for protective antibody. *Lancet* **332:** 263–266.
112. **Matthews, R. C., J. P. Burnie, D. Howat, T. Rowland, and F. Walton.** 1991. Autoantibody to HSP90 can mediate protection against systemic candidosis. *Immunology* **74:**20–24.
113. **Matthews, R. C., G. Rigg, S. Hodgetts, T. Carter, C. Chapman, C. Gregory, C. Illidge, and J. Burnie.** 2003. Preclinical assessment of the efficacy of Mycograb, a human recombinant antibody against fungal Hsp90. *Antimicrob Agents Chemother.* **47:**2208–2216.
114. **Menon, V., D. Li, N. Chauhan, R. Rajnarayanan, A. Dubrovska, A. H. West, and R. A. Calderone.** 2006. Functional studies of the Ssk1p response regulator protein of *Candida albicans* as determined by phenotypic analysis of receiver domain point mutants. *Mol. Microbiol.* **62:** 997–1013.
115. **Momose, Y., and H. Iwahashi.** 2001. Bioassay of cadmium using a DNA microarray: genome-wide expression patterns of *Saccharomyces cerevisiae* response to cadmium. *Environ. Toxicol. Chem.* **20:**2353–2360.
116. **Morgan, B. A., G. R. Banks, W. M. Toone, D. Raitt, S. Kuge, and L. H. Johnston.** 1997. The Skn7 response regulator controls gene expression in the oxidative stress response of the budding yeast *Saccharomyces cerevisiae*. *EMBO J.* **16:**1035–1044.
117. **Moskvina, E., C. Schuller, C. T. C. Maurer, W. H. Mager, and H. Ruis.** 1998. A search in the genome of *Saccharomyces cerevisiae* for genes regulated via stress response elements. *Yeast* **14:**1041–1050.
118. **Munro, C. A., S. Selvaggini, I. de Bruijn, L. Walker, M. D. Lenardon, B. Gerssen, S. Milne, A. J. P. Brown, and N. A. R. Gow.** 2007. The PKC, HOG and Ca2+ signalling pathways coordinately regulate chitin synthesis in *Candida albicans*. *Mol. Microbiol.* **63:** 1399–1413.
119. **Murad, A. M. A., P. Leng, M. Straffon, J. Wishart, S. Macaskill, D. MacCallum, N. Schnell, D. Talibi, D. Marechal, F. Tekaia, C. d'Enfert, C. Gaillardin, F. C. Odds, and A. J. P. Brown.** 2001. *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. *EMBO J.* **20:**4742–4752.
120. **Murad, A. M. A., C. d'Enfert, C. Gaillardin, H. Tournu, F. Tekaia, D. Talibi, D. Marechal, V. Marchais, J. Cottin, and A. J. P. Brown.** 2001. Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors, CaTup1, CaMig1 and CaNrg1. *Mol. Microbiol.* **42:**981–993.
121. **Muzzey, D., C. A. Gomez-Uribe, J. T. Mettetal, and A. van Oudenaarden.** 2009. A systems-level analysis of perfect adaptation in yeast osmoregulation. *Cell* **138:** 160–171.
122. **Nagahashi, S., T. Mio, N. Ono, T. Yamada-Okabe, M. Arisawa, H. Bussey, and H. Yamada-Okabe.** 1998. Isolation of *CaSLN1* and *CaNIK1*, the genes for osmosensing histidine kinase homologues, from the pathogenic fungus *Candida albicans*. *Microbiology* **144:**425–432.
123. **Nasution, O., K. Srinivasa, M. Kim, Y.J. Kim, W. Kim, W. Jeong, and W. Choi.** 2008. Hydrogen peroxide induces hyphal differentiation in *Candida albicans*. *Eukaryot. Cell* **7:**2008–2011.
124. **Navarro-Garcia, F., M. Sanchez, J. Pla, and C. Nombela.** 1995. Functional characterization of the *MKC1* gene of *Candida albicans*, which encodes a mitogen-activated protein kinase homolog related to cell integrity. *Mol. Cell. Biol.* **15:**2197–2206.
125. **Navarro-Garcia, F., R. Alonso-Monge, H. Rico, J. Pla, R. Sentandreu, and C. Nombela.** 1998. A role for the MAP kinase gene *MKC1* in cell wall construction and morphological transitions in *Candida albicans*. *Microbiology* **144:**411–424.
126. **Navarro-Garcia, F., B. Eisman, S. M. Fiuza, C. Nombela, and J. Pla.** 2005. The MAP kinase Mkc1p is activated under different stress conditions in *Candida albicans*. *Microbiology* **151:**2737–2749.

127. **Nguyen, A. N., A. Lee, W. Place, and K. Shiozaki.** 2000. Multistep phosphorelay proteins transmit oxidative stress signals to the fission yeast stress-activated protein kinase. *Mol. Biol. Cell* **11:**1169–1181.
128. **Nicholls, S., M. Straffon, B. Enjalbert, A. Nantel, S. Macaskill, M. Whiteway, and A. J. P. Brown.** 2004. Msn2/4-like transcription factors play no obvious roles in the stress responses of the fungal pathogen, *Candida albicans*. *Eukaryot. Cell* **3:**1111–1123.
129. **Nicholls, S., M. Leach, C. Priest, and A. J. P. Brown.** 2009. Role of the heat shock transcription factor, Hsf1, in a major fungal pathogen that is obligately associated with warm-blooded animals. *Mol. Microbiol.* **74:**844–861.
130. **Nicholls, S., D. M. MacCallum, F. A. R. Kaffarnik, L. Selway, S. C. Peck, and A. J. P. Brown.** 2011. Activation of the heat shock transcription factor Hsf1 is essential for the full virulence of the fungal pathogen *Candida albicans*. *Fungal Genet. Biol.* **48:**297–305.
131. **Nikolaou, E., I. Agrafioti, M. Stumpf, J. Quinn, I. Stansfield, and A. J. P. Brown.** 2009. Phylogenetic diversity of stress signalling pathways in fungi. *BMC Evol. Biol.* **9:**44.
132. **Norbeck, J., A. K. Pahlman, N. Akhtar, A. Blomberg, and L. Adler.** 1996. Purification and characterization of two isoenzymes of DL-glycerol-3-phosphatase from *Saccharomyces cerevisiae*. Identification of the corresponding *GPP1* and *GPP2* genes and evidence for osmotic regulation of Gpp2p expression by the osmosensing mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* **271:**13875–13881.
133. **Nwaka, S., and H. Holzer.** 1998. Molecular biology of trehalose and the trehalases in the yeast *Saccharomyces cerevisiae*. *Prog. Nucleic Acid Res. Mol. Biol.* **58:**197–237.
134. **Odds, F. C.** 1988. Candida *and Candidosis*, 2nd ed. Bailliere Tindall, London, United Kingdom.
135. **Odds, F. C.** 1994. *Candida* species and virulence. *ASM News* **60:**313–318.
136. **Pahlman, A. K., K. Granath, R. Ansell, S. Hohmann, and L. Adler.** 2001. The yeast glycerol 3-phosphatases Gpp1p and Gpp2p are required for glycerol biosynthesis and differentially involved in the cellular responses to osmotic, anaerobic, and oxidative stress. *J. Biol. Chem.* **276:**3555–3563.
137. **Panaretou, B., K. Sinclair, C. Prodromou, J. Johal, L. Pearl, and P. W. Piper.** 1999. The Hsp90 of *Candida albicans* can confer Hsp90 functions in *Saccharomyces cerevisiae*: a potential model for the processes that generate immunoprotective fragments of this molecular chaperone in *C. albicans* infections. *Microbiology* **145:**3455–3463.
138. **Paravicini, G., A. Mendoza, B. Antonsson, M. Cooper, C. Losberger, and M. A. Payton.** 1996. The *Candida albicans PKC1* gene encodes a protein kinase C homolog necessary for cellular integrity but not dimorphism. *Yeast* **12:**741–756.
139. **Phillips, A. J., I. Sudbery, and M. Ramsdale.** 2003. Apoptosis induced by environmental stresses and amphotericin B in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **100:**14327–14332.
140. **Phillips, A. J., J. D. Crowe, and M. Ramsdale.** 2006. Ras pathway signaling accelerates programmed cell death in the pathogenic fungus *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **103:**726–731.
141. **Piekarska, K., E. Mol, M. van den Berg, G. Hardy, J. van den Burg, C. van Roermund, D. MacCallum, F. C. Odds, and B. Distel.** 2006. Peroxisomal fatty acid β-oxidation is not essential for virulence of *Candida albicans*. *Eukaryot. Cell* **5:**1847–1856.
142. **Pinjon, E., D. Sullivan, I. Salkin, D. Shanley, and D. Coleman.** 1998. Simple, inexpensive, reliable method for differentiation of *Candida dubliniensis* from *Candida albicans*. *J. Clin. Microbiol.* **36:**2093–2095.
143. **Piper, P., C. O. Calderon, K. Hatzixanthis, and M. Mollapour.** 2001. Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* **147:**2635–2642.
144. **Posas, F., and H. Saito.** 1997. Osmotic activation of the HOG MAPK pathway via Ste11p MAPKKK: scaffold role of Pbs2p MAPKK. *Science* **276:**1702–1705.
145. **Posas, F., and H. Saito.** 1998. Activation of the yeast SSK2 MAP kinase kinase kinase by the SSK1 two-component response regulator. *EMBO J.* **17:**1385–1394.
146. **Posas, F., S. M. Wurgler-Murphy, T. Maeda, E. A. Witten, T. C. Thai, and H. Saito.** 1996. Yeast HOG1 MAP kinase cascade is regulated by a multistep phosphorelay mechanism in the SLN1-YPD1-SSK1 "two-component" osmosensor. *Cell* **86:**865–875.
147. **Quinn, J., V. J. Findlay, K. Dawson, J. B. Millar, N. Jones, B. A. Morgan, and W. M. Toone.** 2002. Distinct regulatory proteins control the graded transcriptional response to increasing H_2O_2 levels in fission yeast *Schizosaccharomyces pombe*. *Mol. Biol. Cell* **13:**805–816.
148. **Raitt, D. C., A. L. Johnson, A. M. Erkine, K. Makino, B. Morgan, D. S. Gross, and L. H. Johnston.** 2000. The Skn7 response regulator of *Saccharomyces cerevisiae* interacts with Hsf1 *in vivo* and is required for the induction of heat shock genes by oxidative stress. *Mol. Biol. Cell* **11:**2335–2347.
149. **Ramsdale, M., L. Selway, D. Stead, J. Walker, Z. Yin, S. M. Nicholls, E. M. Shiels, and A. J. P. Brown.** 2008. The novel gene *MNL1* regulates weak acid induced stress responses of the fungal pathogen *Candida albicans*. *Mol. Biol. Cell* **19:**4393–4403.
150. **Rauceo, J. M., J. R. Blankenship, J. J. Hamaker, J. S. Deneault, F. L. Smith, A. Nantel, and A. P. Mitchell.** 2008. Regulation of the *Candida albicans* cell wall damage response by transcription factor Sko1 and PAS kinase Psk1. *Mol. Biol. Cell* **19:**2741–2751.
151. **Rementeria, A., R. Garcia-Tobalina, and M. J. Sevilla.** 1995. Nitric oxide-dependent killing of *Candida albicans* by murine peritoneal cells during an experimental infection. *FEMS Immunol. Med. Microbiol.* **11:**157–162.
152. **Rodaki, A., I. M. Bohovych, B. Enjalbert, T. Young, F. C. Odds, N. A. R. Gow, and A. J. P. Brown.** 2009. Glucose promotes stress resistance in the fungal pathogen, *Candida albicans*. *Mol. Biol. Cell* **20:**4845–4855.
153. **Roetzer, A., A. Gregori, A. M. Jennings, J. Quintin, D. Ferrandon, G. Butler, K. Kuchler, G. Ammerer, and C. Schuller.** 2008. *Candida glabrata* environmental stress response involves *Saccharomyces cerevisiae* Msn2/4 orthologous transcription factors. *Mol. Microbiol.* **69:**603–620.
154. **Roman, E., C. Nombela, and J. Pla.** 2005. The Sho1 adaptor protein links oxidative stress to morphogenesis and cell wall biosynthesis in the fungal pathogen *Candida albicans*. *Mol. Cell. Biol.* **25:**10611–10627.
155. **Roman, E., D. M. Arana, C. Nombela, R. Alonso-Monge, and J. Pla.** 2007. MAP kinase pathways as regulators of fungal virulence. *Trends Microbiol.* **15:**181–190.
156. **Roman, E., R. Alonso-Monge, Q. Gong, D. Li, R. A. Calderone, and J. Pla.** 2009. The Cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen *Candida albicans*. *FEMS Yeast Res.* **9:**942–955.
157. **Roman, E., F. Cottier, J. F. Ernst, and J. Pla.** 2009. Msb2 signaling mucin controls activation of Cek1 mitogen-activated protein kinase in *Candida albicans*. *Eukaryot. Cell* **8:**1235–1249.

158. **Rossignol, T., P. Lechat, C. Cuomo, Q. Zeng, I. Moszer, and C. d'Enfert.** 2008. CandidaDB: a multi-genome database for *Candida* species and related *Saccharomycotina*. *Nucleic Acids Res*. **36:**D557–D561.
159. **Rubin-Bejerano, I., I. Fraser, P. Grisafi, and G. R. Fink.** 2003. Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **100:** 11007–11012.
160. **Saijo, T., T. Miyazaki, K. Izumikawa, T. Mihara, T. Takazono, K. Kosai, Y. Imamura, M. Seki, H. Kakeya, Y. Yamamoto, K. Yanagihara, and S. Kohno.** 2010. Skn7p is involved in oxidative stress response and virulence of *Candida glabrata*. *Mycopathologia* **169:**81–90.
161. **Sandini, S., R. Melchionna, C. Bromuro, and R. La Valle.** 2002. Gene expression of 70 kDa heat shock protein of *Candida albicans*: transcriptional activation and response to heat shock. *Med. Mycol.* **40:**471–478.
162. **San Jose, C., R. A. Monge, R. Perez-Diaz, J. Pla, and C. Nombela.** 1996. The mitogen-activated protein kinase homolog *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*. *J. Bacteriol.* **178:**5850–5852.
163. **Santos, J. L., and K. Shiozaki.** 2001. Fungal histidine kinases. *Sci. STKE* **2001:**re1.
164. **Schmitt, A. P., and K. McEntee.** 1996. Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **93:**5777–5782.
165. **Selitrennikoff, C. P., L. Alex, T. K. Miller, K. V. Clemons, M. I. Simon, and D. A. Stevens**. 2001. COS-1, a putative two-component histidine kinase of *Candida albicans*, is an *in vivo* virulence factor. *Med. Mycol.* **39:**69–74.
166. **Sellam, A., C. Askew, E. Epp, H. Lavoie, M. Whiteway, and A. Nantel.** 2009. Genome-wide mapping of the coactivator Ada2p yields insight into the functional roles of SAGA/ADA complex in *Candida albicans*. *Mol. Biol. Cell* **20:**2389–2400.
167. **Shapiro, R., P. Uppuluri, A. K. Zaas, C. Collins, H. Senn, J. R. Perfect, J. Heitman, and L. E. Cowen.** 2009. Hsp90 orchestrates temperature-dependent *Candida albicans* morphogenesis via Ras1-PKA signalling. *Curr. Biol.* **19:**1–9.
168. **Shi, Q. M., Y. M. Wang, X. D. Zheng, R. T. Lee, and Y. Wang.** 2007. Critical role of DNA checkpoints in mediating genotoxic-stress-induced filamentous growth in *Candida albicans*. *Mol. Biol. Cell* **18:**815–826.
169. **Singer, M. A., and S. Lindquist.** 1998. Thermotolerance in *Saccharomyces cerevisiae*: the yin and yang of trehalose. *Trends Biotechnol.* **16:**460–468.
170. **Singh, P., N. Chauhan, A. Ghosh, F. Dixon, and R. A. Calderone.** 2004. *SKN7* of *Candida albicans*: mutant construction and phenotype analysis. *Infect. Immun.* **72:**2390–2394.
171. **Skrzypek, M. S., M. B. Arnaud, M. C. Costanzo, D. O. Inglis, P. Shah, G. Binkley, S. R. Miyasato, and G. Sherlock.** 2010. New tools at the *Candida* Genome Database: biochemical pathways and full-text literature search. *Nucleic Acids Res*. **38:**D428–D432.
172. **Smith, D. A., S. Nicholls, B. A. Morgan, A. J. P. Brown, and J. Quinn.** 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. *Mol. Biol. Cell* **15:** 4179–4190.
173. **Smith, D. A., B. A. Morgan, and J. Quinn.** 2010. Stress signalling to fungal stress-activated protein kinase pathways. *FEMS Microbiol. Lett*. **306:**1–8.
174. **Sorger, P. K., and H. R. B. Pelham.** 1988. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* **54:**855–864.
175. **Srikantha, T., L. Tsai, K. Daniels, L. Enger, K. Highley, and D. R. Soll.** 1998. The two-component hybrid kinase regulator CaNIK1 of *Candida albicans*. *Microbiology* **144:** 2715–2729.
176. **Stohs, S. J., and D. Bagchi.** 1995. Oxidative mechanisms in the toxicity of metal ions. *Free Radic. Biol. Med.* **18:**321–336.
177. **Swoboda, R., S. Miyasaki, D. Greenspan, and J. S. Greenspan.** 1993. Heat-inducible ATP-binding proteins of *Candida albicans* are recognized by sera of infected patients. *J. Gen. Microbiol.* **139:**2995–3003.
178. **Swoboda, R. K., G. Bertram, H. Hollander, D. Greenspan, J. S. Greenspan, N. A. R. Gow, G. W. Gooday, and A. J. P. Brown.** 1993. Glycolytic enzymes of *Candida albicans* are nonubiquitous immunogens during candidiasis. *Infect. Immun.* **61:**4263–4271.
179. **Swoboda, R. K., G. Bertram, S. Delbruck, J. F. Ernst, N. A. R. Gow, G. W. Gooday, and A. J. P. Brown.** 1994. Fluctuations in glycolytic mRNA levels during the yeast-to-hyphal transition in *Candida albicans* reflect underlying changes in growth and are not a response to cellular dimorphism. *Mol. Microbiol.* **13:**663–672.
180. **Swoboda, R. K., G. Bertram, S. Budge, G. W. Gooday, N. A. R. Gow, and A. J. P. Brown.** 1995. Structure and regulation of the *HSP90* gene from the pathogenic fungus *Candida albicans*. *Infect. Immun.* **63:**4506–4514.
181. **Tamas, M. J., K. Luyten, F. C. W. Sutherland, A. Hernandez, J. Albertyn, H. Valadi, H. Li, B. A. Prior, S. G. Kilian, J. Ramos, L. Gustafsson, J. M. Thevelein, and S. Hohmann.** 1999. Fps1p controls the accumulation and release of the compatible solute glycerol in yeast osmoregulation. *Mol. Microbiol.* **31:**1087–1104.
182. **Tatebayashi, K., K. Tanaka, H. Y. Yang, K. Yamamoto, Y. Matsushita, T. Tomida, M. Imai, and H. Saito.** 2007. Transmembrane mucins Hkr1 and Msb2 are putative osmosensors in the SHO1 branch of yeast HOG pathway. *EMBO J.* **26:**3521–3533.
183. **Thevelein, J. M., L. Cauwenberg, S. Colombo, J. H. De Winde, M. Donation, F. Dumortier, L. Kraakman, K. Lemaire, P. Ma, D. Nauwelaers, F. Rolland, A. Teunissen, P. Van Dijck, M. Versele, S. Wera, and J. Winderickx.** 2000. Nutrient-induced signal transduction through the protein kinase A pathway and its role in the control of metabolism, stress resistance, and growth in yeast. *Enzyme Microb. Technol.* **26:**819–825.
184. **Thewes, S., M. Kretschmar, H. Park, M. Schaller, S. G. Filler, and B. Hube.** 2007. *In vivo* and *ex vivo* comparative transcriptional profiling of invasive and noninvasive *Candida albicans* isolates identifies genes associated with tissue invasion. *Mol. Microbiol.* **63:** 1606–1628.
185. **Toone, W. M., and N. Jones.** 1998. Stress-activated signalling pathways in yeast. *Genes Cells* **3:**485–498.
186. **Toone, W. M., B. A. Morgan, and N. Jones.** 2001. Redox control of AP-1-like factors in yeast and beyond. *Oncogene* **20:**2336–2346.
187. **Torosantucci, A., P. Chiani, F. De Bernardis, A. Cassone, J. A. Calera, and R. A. Calderone.** 2002. Deletion of the two-component histidine kinase gene (*CHK1*) of *Candida albicans* contributes to enhanced growth inhibition and killing by human neutrophils in vitro. *Infect. Immun.* **70:**985–987.
188. **Ullmann, B. D., H. Myers, W. Chiranand, A. L. Lazzell, Q. Zhao, L. A. Vega, J. L. Lopez-Ribot, P. R. Gardner, and M. C. Gustin.** 2004. Inducible defense mechanism against nitric oxide in *Candida albicans*. *Eukaryot. Cell* **3:**715–723.

189. **Urban, C., X. Xiong, K. Sohn, K. Schroppel, H. Brunner, and S. Rupp.** 2005. The moonlighting protein Tsa1p is implicated in oxidative stress response and in cell wall biogenesis in *Candida albicans*. *Mol. Microbiol.* **57:** 1318–1341.
190. **Van Dijck, P., L. De Rop, K. Szlufcik, E. Van Ael, and J. M. Thevelein.** 2002. Disruption of the *Candida albicans TPS2* gene encoding trehalose-6-phosphate phosphatase decreases infectivity without affecting hypha formation. *Infect. Immun.* **70:**1772–1782.
191. **Vazquez-Torres, A., J. Jones-Carson, and E. Balish.** 1996. Peroxynitrite contributes to the candidacidal activity of nitric oxide-producing macrophages. *Infect. Immun.* **64:**3127–3133.
192. **Veal, E. A., A. M. Day, and B. A. Morgan.** 2007. Hydrogen peroxide sensing and signalling. *Mol. Cell* **26:**1–14.
193. **Veal, E. A., V. J. Findlay, A. M. Day, S. M. Bozonet, J. M. Evans, J. Quinn, and B. A. Morgan.** 2004. A 2-Cys peroxiredoxin regulates peroxide-induced oxidation and activation of a stress-activated MAP kinase. *Mol. Cell* **15:**129–139.
194. **Vido, K., D. Spector, G. Lagniel, S. Lopez, M. B. Toledano, and J. A. Labarre.** 2001. Proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **276:**8469–8474.
195. **Vylkova, S., W. S. Jang, W. Li, N. Nayyar, and M. Edgerton.** 2007. Histatin 5 initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway. *Eukaryot. Cell* **6:**1876–1888.
196. **Walker, L. A., C. A. Munro, I. de Brujin, M. D. Lenardon, A. McKinnon, and N. A. R. Gow.** 2008. Stimulation of chitin synthesis rescues *Candida albicans* from echinocandins. *PLoS Pathog.* **4:**e1000040.
197. **Walker, L. A., D. M. MacCallum, G. Bertram, N. A. R. Gow, F. C. Odds, and A. J. P. Brown.** 2009. Genome-wide analysis of *Candida albicans* gene expression patterns during infection of the mammalian kidney. *Fungal Genet. Biol.* **46:**210–219.
198. **Wang, Y., Y. Y. Cao, X. M. Jia, Y. B. Cao, P. H. Gao, X. P. Fu, K. Ying, W. S. Chen, and Y. Y. Jiang.** 2006. Cap1p is involved in multiple pathways of oxidative stress response in *Candida albicans*. *Free Radic. Biol. Med.* **40:** 1201–1209.
199. **Whiteway, M., D. Dignard, and D. Y. Thomas.** 1992. Dominant negative selection of heterologous genes: isolation of *Candida albicans* genes that interfere with *Saccharomyces cerevisiae* mating factor-induced cell cycle arrest. *Proc. Natl. Acad. Sci. USA* **89:**9410–9414.
200. **Whiteway, M.** 2000. Transcriptional control of cell type and morphogenesis in *Candida albicans*. *Curr. Opin. Microbiol.* **3:**582–588.
201. **Wiederrecht, G., D. Seto, and C. S. Parker.** 1988. Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. *Cell* **54:**841–853.
202. **Wilson, D., A. Tutulan-Cunita, W. Jung, N. C. Hauser, R. Hernandez, T. Williamson, K. Piekarska, S. Rupp, T. Young, and L. Stateva.** 2007. Deletion of the high-affinity cAMP phosphodiesterase encoded by *PDE2* affects stress responses and virulence in *Candida albicans*. *Mol. Microbiol.* **65:**841–856.
203. **Wolfe, K. H., and D. C. Shields.** 1997. Molecular evidence for an ancient duplication of the entire yeast genome. *Nature* **387:**708–713.
204. **Wysong, D. R., L. Christin, A. M. Sugar, P. W. Robbins, and R. D. Diamond.** 1998. Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. *Infect. Immun.* **66:** 1953–1961.
205. **Yamada-Okabe, T., T. Mio, N. Ono, Y. Kashima, M. Matsui, M. Arisawa, and H. Yamada-Okabe.** 1999. Roles of three histidine kinase genes in hyphal development and virulence of the pathogenic fungus *Candida albicans*. *J. Bacteriol.* **181:**7243–7247.
206. **Yamamoto, A., Y. Mizukami, and H. Sakurai.** 2005. Identification of a novel class of target genes and a novel type of binding sequence of heat shock transcription factor in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280:** 11911–11919.
207. **Yin, Z., D. Stead, J. Walker, L. Selway, D. Smith, A. J. P. Brown, and J. Quinn.** 2009. A proteomic analysis of the salt, cadmium and peroxide stress responses in *Candida albicans* and the role of the Hog1 SAPK in regulating the stress-induced proteome. *Proteomics* **9:**4686–4703.
208. **Zakikhany, K., J. R. Naglik, A. Schmidt-Westhausen, G. Holland, M. Schaller, and B. Hube.** 2007. *In vivo* transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. *Cell. Microbiol.* **9:**2938–2954.
209. **Zaragoza, O., M. A. Blázquez, and C. Gancedo.** 1998. Disruption of the *Candida albicans TPS1* gene encoding trehalose-6-phosphate synthase impairs formation of hyphae and decreases virulence. *J. Bacteriol.* **180:** 3809–3815.
210. **Zeuthen, M. L., and D. H. Howard.** 1989. Thermotolerance and the heat-shock response in *Candida albicans*. *J. Gen. Microbiol.* **135:**2509–2518.
211. **Zhang, X., M. De Micheli, S. T. Coleman, D. Sanglard, and W. S. Moye-Rowley.** 2000. Analysis of the oxidative stress regulation of the *Candida albicans* transcription factor, Cap1p. *Mol. Microbiol.* **36:**618–629.
212. **Zhao, X., R. Mehrabi, and J. R. Xu.** 2007. Mitogen-activated protein kinase pathways and fungal pathogenesis. *Eukaryot. Cell* **6:**1701–1714.
213. **Zhao, X. J., D. Raitt, P. V. Burke, A. S. Clewell, K. E. Kwast, and R. O. Poyton.** 1996. Function and expression of flavohemoglobin in *Saccharomyces cerevisiae*. Evidence for a role in the oxidative stress response. *J. Biol. Chem.* **271:**25131–25138.
214. **Znaidi, S., K. S. Barker, S. Weber, A. M. Alarco, T. T. Liu, G. Boucher, P. D. Rogers, and M. Raymond.** 2009. Identification of the *Candida albicans* Cap1p regulon. *Eukaryot. Cell* **8:**806–820.

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch16

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Adhesins in Opportunistic Fungal Pathogens

REBECCA ZORDAN AND BRENDAN CORMACK

ADHESINS IN FUNGI

In fungi, the cell wall has multiple roles, providing structural support to the cell but also forming the interface between the yeast and its environment. In pathogenic fungi, proteins of the cell wall mediate primary interactions with host cells, allowing adherence to and in some cases invasion of host cells. The cell wall is also a primary target of the immune system. As a result of these diverse aspects of cell wall function, fungi have evolved a diverse array of proteins that function in the cell wall. Cell wall proteins (CWPs) are highly variable among fungi, and even among fungal isolates, likely reflecting adaptation to different environments and microevolution in response to selective pressure. This chapter is concerned with cell surface proteins in two *Candida* species, *Candida albicans* and *Candida glabrata*, and in their nonpathogenic relative *Saccharomyces cerevisiae*, specifically focusing on the diversity and function of proteins implicated in adherence of the yeasts to each other or to other surfaces, including host cells.

Cell Wall Structure

The cell wall is composed of mannoproteins, β-glucans, and chitin. Branched β-1,3-glucan polymers provide the major structural support in the cell wall, and these glucans are covalently bound to β-1,6-glucans and chitin, a linear polymer of β-1,4-GlcNAc. CWPs coat the outermost surface of the cell wall, providing a permeability barrier and a mannosylated coat (23). While it is beyond the scope of this review, a key function of this outer protein coat is to mask the structural glucans from the host immune system, which has dedicated receptors to recognize these polysaccharides in the cell wall (129, 130). There are two major classes of CWPs, defined by their mode of attachment to the cell wall. The most abundant CWPs are glycosylphosphatidylinositol (GPI)-modified CWPs (GPI-CWPs), which are attached via a covalent linkage between a remnant of the GPI anchor and β-1,6-glucans in the cell wall (56, 60, 71). In line with this covalent linkage, GPI-CWPs can be released from cells using β-glucanase digestion or by pyridine/hydrofluoric acid treatment that cleaves the GPI anchor; the covalent forms are not released by other, less disruptive treatments like sodium dodecyl sulfate, treatment with reducing agents, or boiling (21). The other major class of CWPs consists of the alkali-sensitive linkage CWPs (ASL-CWPs), which include the Pir protein family. These can be released from cells using β-glucanase or mild alkali treatment. The Pir proteins do not have a GPI anchor and instead are attached via links between mannose residues decorating the Pir proteins and β-1,3-glucans in the cell wall (reviewed in reference 59). While this understanding of CWP attachment is largely derived from analysis of the *S. cerevisiae* cell wall, there are excellent data that the cell walls of other ascomycetes share this basic structure: in silico identification of putative CWPs identified ASL-CWPs as well as 75 to 100 GPI-CWPs in *C. albicans* and *C. glabrata*, respectively (20, 128), and proteomic analysis of the covalently cross-linked, glucanase-releasable protein complement of *C. glabrata* and *C. albicans* shows the presence of the predicted GPI-CWPs in isolated cell wall fractions (19, 21).

This chapter focuses in particular on those cell surface proteins that enable yeasts to adhere to substrates and other cells. In *C. albicans* and *C. glabrata*, the majority of characterized proteins with function in adherence are GPI-CWPs, which can be classified into a number of protein families. This chapter also includes a brief discussion of adherence and evolution of adhesins in the nonpathogenic yeast *Saccharomyces cerevisiae*. By mediating a variety of physical interactions, adhesins play a role in multiple processes in fungal growth and infection, including adherence to host cells in both commensal and pathogenic settings, adherence to abiotic surfaces, and growth in communities, such as biofilms, requiring adhesion between yeast cells as well as adherence to the biofilm substrate. The presence of large families of adhesin proteins suggests substantial evolutionary pressure for diversification of adhesin genes, to optimize substrate specificity and as a mode of adaptation to the diverse environments that fungi, particularly pathogenic fungi, encounter.

Structure of GPI-Anchored Adhesins

In this section, we discuss general structure of *Candida* and *S. cerevisiae* adhesins, illustrated by reference to two large families of adhesins: those corresponding to the *ALS* genes

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of *C. albicans* and those corresponding to the *EPA* genes of *C. glabrata*. More detailed information on individual members of the two families is presented later in the chapter.

Figure 1 and Color Plate 6 illustrate the modular domain structures of the Als and Epa adhesin families, respectively, and are described in detail below. Many of the structural features are also found in the *C. albicans* adhesins Hwp1 and Eap1 (Fig. 2). To construct Fig. 1 and 2 and Color Plate 6, many domains were defined by sequence analysis performed for this chapter; they may differ slightly from previously published analyses due to strain differences, sequence source, or sequence definitions of the repeat units. *C. albicans* protein sequence was derived from haploid assembly 21 of the *C. albicans* SC5314 genome (*Candida* Genome Database, accessed August 2010 [107]).

In general, yeast adhesins have a conserved overall structure: a secretion signal sequence at the N terminus of the protein, followed by a ligand-binding domain, then by a Ser- and Thr- rich region, and lastly by a C-terminal hydrophobic signal for GPI anchor addition in the endoplasmic reticulum. The N-terminal signal sequence is required for secretion, and the C-terminal GPI anchor signal is required for expression and cross-linking at the cell surface. Initially, the GPI-anchored proteins traffic through the normal secretory pathway to the plasma membrane; there, the GPI anchor is processed and the adhesin ultimately is linked through a remnant of the GPI anchor to β-1,6-glucan. Thus, mature adhesins are covalently attached to the carbohydrates of the cell wall through a remnant of the GPI anchor (58, 64, 71). Loss of the C-terminal hydrophobic ω sequence causes the adhesin proteins to be secreted into the environment, rather than linked to the plasma membrane or cell wall (31, 113).

The protein sequences obtained for the Hwp1, Eap2, and most Als proteins contain a signal sequence for secretion at their N termini and hydrophobic sequences for attachment of GPI anchors at their C termini, though they are not represented in Fig. 1 and 2 (GPI-SOM online tool [26]). The only exception is the Als2 amino acid sequence from assembly 21, which does not contain a GPI attachment signal, hinting that this sequence may be inaccurate, because Als2 has been detected in the cell wall of *C. albicans* SC5314 (18).

While other portions of the adhesins contribute to general adherence, the N-terminal domain is thought to be primarily responsible for specific interactions with ligands (44). As a rule, the N-terminal domain is roughly 300 amino acids in length and in the case of several adhesins has been shown to be a major contributor to adherence specificity. For example, in the *ALS* genes, this domain, which is modeled to form tandem immunoglobulin-like (Ig-like) domains (49, 105), is a major contributor to specificity: chimeric proteins made by switching the Ig regions of Als5 and Als6 possessed binding preferences corresponding to the Als from which the Ig region originated (105). For the *EPA* genes, the isolated N-terminal domain is sufficient to mediate adherence with identical specificity to the full-length protein, again consistent with this being the major contributor to ligand specificity (31, 140). *C. albicans* Als proteins also contain a Thr-rich (T) region, whose sequence is highly conserved among most Als proteins and which lies between the Ig-like globular domains and the Ser/Thr region (90). The role of the T region has been studied primarily in Als5 and is discussed in detail below.

The Ser/Thr regions of fungal adhesins vary dramatically in length among different adhesins and also among different alleles of the same adhesin. It is the site of attachment for many carbohydrate modifications: Ser and Thr residues are O glycosylated, and the abundant glycosylation forces this domain into an extended conformation. Data from several systems suggest that the Ser/Thr region serves as a structural stalk to extend the N-terminal ligand-binding domain to the cell surface: when expressed in *S. cerevisiae*, mutant versions of the *C. glabrata* adhesin Epa1 lacking the Ser/Thr repeat domain are processed correctly and incorporated into the cell wall, but the N-terminal binding domain is buried in the cell wall rather than being displayed on the surface of the cell (31). In *C. albicans*, adherence mediated by Als3 (see below) is higher for alleles containing longer Ser/Thr domains, consistent with more efficient presentation of the N-terminal Ig region on the cell surface (89, 134). Additional evidence for a role of the stalk region in projecting the N-terminal domain into the environment comes from analysis of *S. cerevisiae* flocculins encoded by the *FLO* genes, discussed in detail below. Expression of these proteins on the surface of *S. cerevisiae* results both in adherence to inert substrates and in clumping, or flocculation, of the cells. These functions of the flocculins are modulated in direct proportion to the length of the Ser/Thr-rich region (124), again consistent with a role for the length of the Ser/Thr-rich region in adhesin function.

In each Als protein in *C. albicans*, a portion of the Ser/Thr stalk is composed of a series of tandemly repeated 36-amino-acid units. The number of repeat units in these tandem repeat domains (TRDs), may vary among Als proteins, strains, and even alleles for the same Als protein in a given strain (74, 132, 134) (Fig. 1). The precise sequences of the 36-mer repeat units vary slightly in the TRDs, and Als proteins can be classified based on the sequences found in this region (reviewed in reference 45). As described above, the length of the Ser/Thr stalk is correlated with adhesive functions in a number of adhesins; increasing or decreasing the number of repeat units in the TRD will affect the length of the stalk and thus the adhesive properties of the Als proteins (89, 134). Though the TRD with 36-mer repeat units is characteristic of the Als family of adhesins, other adhesin families also have tandemly repeated sequences of different lengths (for example, see *C. glabrata* Epa discussion below). Variations in the number of tandem repeats in these other adhesins may also alter adhesive properties due to structural lengthening of the Ser/Thr stalks.

In addition to this primary structural function, the TRD in the *ALS* family has been shown to play additional roles. TRDs modulate ligand binding by the N-terminal globular domain, improving binding affinity without altering specificity. Purified truncated versions of Als5, containing the Ig-like domain and conserved T region at the N terminus, display minimal binding to fibronectin, but this binding is greatly improved when TRDs are included in the truncated Als5 protein (30, 97). Similarly, Als1-mediated binding to endothelial cells is reduced when TRDs are removed from the central portion of the protein, even though the N terminus of Als1 is known to be surface expressed (75). Computer modeling and circular-dichroism measurements of a synthetic peptide indicate that the TRDs of Als proteins form antiparallel β-sheet secondary structures (30, 97).

TRDs may also have direct roles in binding. The TRD of Als5 contributes to cell-cell aggregation, even in the absence of the Ig-like domain and the T region (97), suggesting a possible role for TRDs in different Als proteins to mediate cell-cell interaction. Consistent with this notion,

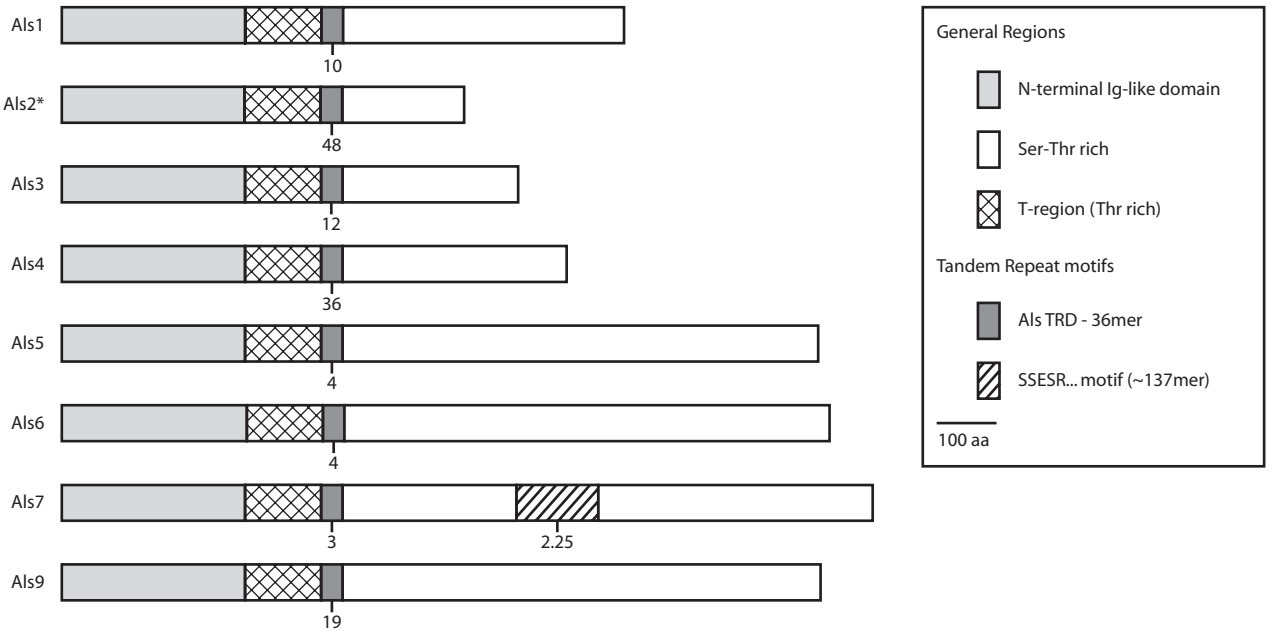

FIGURE 1 Schematic of protein domains in the C. *albicans* Als adhesins. The protein domains and repeat motifs described in the section "Structure of GPI-Anchored Adhesins" are indicated in the key. Each drawing is to scale, with tandem repeats collapsed such that one motif unit is shown and the number of repeat units is indicated below the drawing. The consensus sequence for the Als TRD repeat unit is xNxTVTTTExWSxSxATTTTxTxPPxGTDxVIIxEP (75% identity among 176 variants). Diagonal lines represent the 132- to 137-mer repeats spanning from SSESR to SDE; these have been designated the VASES repeats in previous publications (132). Genes are as follows: *ALS1* (orf19.5741), *ALS2* (orf19.1097), *ALS3* (orf19.1816), *ALS4* (orf19.4555), *ALS5* (orf19.5736), *ALS6* (orf19.7414), *ALS7* (orf19.7400), and *ALS9* (orf19.5742). 10.1128/9781555817176.ch16f1

while purified truncated forms of Als5, containing only the Ig-like globular domain and the T region, saturate when binding limiting amounts of fibronectin, truncated forms of Als5 that include the Ig-like globular domain, the T region, and the TRDs do not saturate when titrated against limiting amounts of fibronectin. This may suggest that the TRDs facilitate a direct interaction with themselves or possibly another domain of the Als protein (30).

Adhesins in *Candida albicans*

The most extensively studied family of adhesins in any *Candida* species is the one corresponding to the C. *albicans ALS* gene family, which includes eight family members (45, 65, 118). Two other GPI-CWPs, not members of the Als family, Hwp1 and Eap1, have also been found to mediate adhesion in C. *albicans* (68, 115, 116).

Als Protein Roles in Epithelial and Endothelial Adherence and Infection Models

Als-mediated adherence to epithelial and endothelial cells and subsequent endocytosis are critical steps during infections, and endocytosis of *Candida* leads to tissue damage (29, 92). A number of studies have examined the role of *ALS* genes in mediating adherence to—and invasion of—cultured mammalian epithelial and endothelial cells. Analysis of the roles of individual Als proteins is complicated by presumed redundancy of function, which limits the phenotypes that can be observed by mutation of single *ALS* genes in C. *albicans*. Nonetheless, several reports have teased out roles for individual *ALS* genes in interaction with host cells.

ALS1. *ALS1* deletion has been shown to have an impact on adherence. Strains lacking *ALS1* suffer a modest (20 to 35%) decrease in adhesion to human umbilical vein endothelial cells (HUVECs) (32, 94, 134). *als1*Δ/*als1*Δ mutants are not compromised for binding to oral epithelial cells or buccal epithelial cells (BECs) (94, 134).

ALS2. Studies of *ALS2* have relied on an *als2*Δ/*pMAL-ALS2* strain, because attempts to make a homozygous deletion mutant have been unsuccessful. In this *als2*Δ/*pMAL-ALS2* strain, *ALS2* expression levels are very low when the cells are grown under noninducing conditions (138). Under noninducing conditions, this strain has subtle defects in adherence to vascular endothelial cells but binds to BECs at wild-type levels in vitro (138). However, the *als2*Δ/*pMAL-ALS2* strain showed reduced adherence and damage in the reconstituted human epithelial (RHE) assay, which may be related to the decreased ability of this mutant strain to form germ tubes (138).

ALS4, ALS5, ALS6,* and *ALS7. *ALS4* mutants have slight defects in binding to vascular endothelial cells but adhere to BECs at wild-type levels. *ALS4* mutants also do not have adherence or damage defects in the RHE model of host tissue damage (138). Deletion of certain *ALS* genes (*ALS5*, *ALS6*, or *ALS7*) resulted in increased adhesion to HUVECs and BECs (135).

ALS9. Als9 allows *C. albicans* to adhere to endothelial cells in vitro, as indicated by the inability of an *als9Δ/als9Δ* to adhere to human vascular endothelial cells (136). *ALS9* is unique within the *ALS* family in that the two alleles present in *C. albicans* encode proteins that differ greatly in their N-terminal domain, with 16% amino acid differences between the two versions (in the N-terminal domain). This has a functional consequence; in complementation assays in an *als9-1Δ/als9-2Δ* strain, expression of Als9-2 but not Als9-1 mediates adherence to human vascular endothelial cell monolayers (136). *als9Δ/als9Δ* mutants do not exhibit a defect in adherence to BECs or to immobilized laminin.

Heterologous Expression of *ALS* Genes

Analysis of single *ALS* mutants is difficult in *C. albicans*, because loss of a single adhesin may not often lead to an altered phenotype, as other family members compensate for the loss of *ALS*. To study Als adhesins in isolation, they have been expressed in *S. cerevisiae*, which does not naturally adhere well to substrates or human tissues. The advantage of this method is that each adhesin's role in adherence may be studied individually. However, such studies are limited by the considerations that heterologously expressed genes may not function properly in a different genetic context, dosage differences may affect function, and any required cofactors or posttranslational modifications may not be available in the heterologous system.

Early studies found that *S. cerevisiae* cells expressing *ALS5* are also able to bind to collagen IV, laminin, and fibronectin, all components of the extracellular matrix (35), and that *S. cerevisiae* cells expressing Als1 are able to bind avidly to endothelial and epithelial cells. In a later, more comprehensive study, *S. cerevisiae* strains expressing *ALS1*, *ALS3*, or *ALS5* were shown to be competent to bind to gelatin, fibronectin, laminin, FaDu epithelial cells, and endothelial cells in vitro. Als6 mediates binding to gelatin, and Als9 to laminin, while *ALS7* expression did not make *S. cerevisiae* competent to bind any of the substrates tested (105). Even for Als1, Als3, and Als5, which enabled cells to bind to all substrates tested, there were significant differences in the patterns of binding for each Als-substrate pair, indicating that individual Als proteins may be adapted to preferentially bind a particular set of ligands, and encoding multiple Als proteins may broaden the range of materials to which *C. albicans* may bind. This difference in substrate specificity is also shown by differential binding of *S. cerevisiae* expressing *ALS1* and *ALS5* to peptides of different sequence (61).

ALS3-Mediated Adherence and Invasion

Als3 has been implicated, more than any other Als adhesin, in specific interactions with host cells. Cells lacking *ALS3* have a strongly reduced ability to adhere to and damage endothelial and epithelial cells (94, 134). Moreover, antibodies specific for Als3 can block adherence of *C. albicans* to endothelial cells and BECs (14). Strikingly, after cultured host cells have been bound by *C. albicans* cells, the host cells endocytose the yeast in a primarily clathrin-dependent manner which relies largely on Als3 on the surface of the yeast, though Als1 and Als5 also mediate low levels of invasion (79, 94, 105). This process is passive from the perspective of the yeast; live and dead *C. albicans* cells invade cultured human cells equally well (92, 93, 103). In vitro assays utilizing beads coated with the N-terminal (ligand binding) portions of Als3 demonstrated that the protein mediates binding to E-cadherin expressed ectopically in CHO cells (94). This meshes nicely with the observation that Als3 promoted binding to endothelial cells (naturally expressing N-cadherin) and FaDu oral epithelial cells (naturally expressing E-cadherin). (94). The ligand-binding domain of Als1 may also interact with cadherins, but to a lesser extent than Als3. Computer models of the protein structure of the Als1 and Als3 N-terminal domains suggest that the protein conformation of Als3 is responsible for the increased interaction with cadherins: predictions of surface hydrophobicity, electrostatic interactions, and interaction energies between polypeptide chains are consistent with Als3 forming a molecular cleft which allows more extensive interactions with cadherins than occur with Als1 (94).

ALS3 and Iron Acquisition

In addition to its role in adherence and invasion, Als3 has been implicated in iron acquisition. Iron acquisition is a critical process during microbial infections. Host cells tightly regulate the amount of free iron available to any microbes by coordinating it with host proteins, including ferritin. These host mechanisms of iron limitation are highly relevant to *C. albicans* infection, since *C. albicans*-induced damage to cultured epithelial monolayers is correlated with the relative iron content in the epithelial cells. Increased iron concentrations lead to increased host cell damage by *C. albicans*; depletion of iron protects the epithelial cells from *C. albicans* invasion and damage (1). Recent studies have found that *C. albicans* is able to grow on media with ferritin as the sole source of iron (1). Hyphal *C. albicans* cells expressing *ALS3*, but not yeast form cells, are able to bind to ferritin.

Als3, then, has the unique position of having roles in three virulence-related processes: host cell binding, invasion, and iron acquisition. Notably, and potentially due to the key role of Als3 and Als1 in tissue adherence, invasion, and (for Als3) iron acquisition, Als1 and Als3 fragments have been shown to be efficacious as vaccines to prevent disseminated *Candida* infections (50, 51, 110–112).

ALS5: Als Structure and Amyloid Formation

Sequences found within the T region (see above) of Als proteins confer the ability to form β-aggregates and amyloid fibers, as suggested by dye binding and electron microscopy (90, 96). Amyloid formation has been analyzed primarily for the Als5 protein, and amyloids have been shown to form with purified portions of Als5 that include the defined T region (90). In silico predictions of aggregation-prone sequences identified clusters of Thr, Val, and Ile residues that lie within the T regions in all Als proteins (90). Similar clusters of Thr, Val, and Ile are also found in non-Als adhesins in *C. albicans* and *S. cerevisiae*, though these proteins do not have a strictly conserved T region (96). Functional protein aggregation behavior has been observed for peptide fragments of Als5, as well as the *C. albicans* adhesin Eap1 and Flo1 and Flo11 flocculins of *S. cerevisiae* (96). Amyloid formation may have a functional role in the context of a yeast cell, since Als5-induced aggregation increases thioflavin T binding and shows surface birefringence, which are both hallmarks of amyloids. Additionally, amyloid binding dyes inhibit cell aggregation (96). Amyloid formation has been analyzed using purified protein or peptide, or by heterologous expression in *S. cerevisiae*, but not in the context of normal expression levels in *C. albicans*. While the role of amyloid formation in adherence of *C. albicans* is therefore still unclear, this intriguing mechanism may play a role in

adherence and interaction between yeast cells expressing different adhesins in response to different environmental cues.

Regulation of *ALS* Expression

Expression levels of individual *ALS* genes vary depending on growth state and morphology, as monitored by measuring transcript and protein abundance. In general it appears that *ALS1*, *ALS2*, and *ALS3* expression is detectable under numerous growth conditions, and expression is highly upregulated when cells are grown in media promoting hyphal growth (YPD plus serum or RPMI) (39). Additionally, *ALS1* is expressed strongly in lag phase, after cells are diluted into fresh medium to induce either yeast or hyphal form growth, with expression levels declining as the cultures enter steady-state log-phase growth (39). *ALS6* and *ALS7* have been found to be expressed at consistently low levels under all conditions tested (135). Expression of *ALS* genes is regulated by multiple signaling pathways, including the cyclic AMP/protein kinase A (cAMP/PKA) pathway (6, 109), in response to nutritional cues. Adhesin genes are also regulated by the TOR pathway, as indicated most clearly by induction of *ALS1*, *ALS3*, and *HWP1* (see below) by exposure to rapamycin. Thus, adhesin expression is regulated by major nutritional sensing pathways in the cell (4).

At the transcriptional level, *ALS* gene expression is primarily controlled by two central regulators, Efg1 and Tup1, which control separate pathways regulating morphogenesis in *C. albicans*. Efg1 is a sequence-specific DNA binding protein that regulates transcription of target genes and induces filamentous growth (66). *efg1Δ/efg1Δ* mutants are unable to form hyphae under most filament-inducing conditions (73, 117). Conversely, Tup1 is a general transcription repressor that works with many sequence-specific DNA binding proteins and functions to repress filamentous growth (108). In many instances, adhesin gene expression is correlated with growth in either the yeast or hyphal state and is directly regulated by Efg1 and/or Tup1. For instance, *HWP1* and *ALS3* are expressed specifically in hyphal cells and transcription of these genes is activated by Efg1 and repressed by Tup1 (2, 48, 53, 55, 115). Efg1 can directly bind to a sequence found in the promoters of *HWP1*, *ALS3*, and *ALS8*, and Efg1 can also activate expression of the hypha-specific adhesins in *C. albicans*, *HWP1* (7), and *EAP1* (68) (see later discussion of the latter genes). Analysis of the *ALS3* promoter has shown that *ALS3* expression is regulated by a number of transcriptional regulators that control morphogenesis, including Efg1, Cph1, Tec1, Bcr1, Nrg1, Rfg1, and Tup1 (2). In yeast form cells, Nrg1 and Rfg1 bind *ALS3* promoter and recruit Tup1 to repress *ALS3* transcription. Tec1 is indirectly required for *ALS3* expression in hyphal cells, via its role in inducing *BCR1* expression (see below) (2).

Als1 is expressed in both yeast and hyphal cells in some *C. albicans* strains (48, 55), and its expression requires Efg1 (32). Additionally, ectopic expression of *ALS1* can partially rescue the filamentation defects in strains lacking Efg1 (32). Strains lacking *ALS1* have a delay in transitioning to hyphal growth under filament-inducing conditions, further linking the role of *ALS1* in filamentous growth and *EFG1* regulation (32). The ability to transition between yeast and hyphal growth states is an important physiological feature in *C. albicans*, and the delay in filamentation seen in the *als1Δ/als1Δ* mutant attenuates its virulence in a mouse model of disseminated candidiasis (32). These results indicate that the growth form of the *Candida* cell and the expression of *ALS* adhesins are tightly linked.

Expression of Als Adhesins during Infection

Given the large number of *ALS* genes in the family and differences in ligand specificity (measured in vitro), individual adhesins may have different roles during infection, with different adhesins allowing *Candida* to adhere to specific host tissues or colonize specific niches. Identification of host sites where particular *ALS* genes are expressed is one way in which specific roles of each *ALS* gene can be analyzed. *ALS* expression has been monitored by reverse transcriptase PCR (RT-PCR) (nonquantitatively) in models of infection and in samples taken from clinical settings. Gene expression was examined in reconstituted vaginal epithelium models, in a mouse model of vaginal infection, and in vaginal samples taken from symptomatic and asymptomatic women, potentially representing *C. albicans* in a pathogenic or commensal state (13). In these studies, all *ALS* family members were detected, though *ALS1*, *ALS2*, *ALS3*, and *ALS9* were the most abundant. *ALS4* expression decreased during early stages of growth in models of vaginal infections (reconstituted vaginal epithelium models or mouse models), but expression increased at later time points. This indicates that *ALS4* transcript levels might increase later during infection due to damage to host tissues or as a result of growth in biofilms on the host tissue that form at later time points (13, 37). *ALS4* expression is higher in *C. albicans* isolates taken from the oral mucosa than in vaginal *C. albicans* specimens, pointing to a potential role for Als4 in the oral cavity (13, 38). Though *ALS4* and *ALS5* transcripts were present at relatively low levels under most conditions tested, they were most abundant in clinical samples taken from asymptomatic patients, perhaps indicating that these adhesins have a role in commensal interactions (13).

Non-Als Adhesins in *C. albicans*

Hwp1. Hwp1 is a non-ALS adhesin in *C. albicans* which has been well studied in the context of hyphal cell expression and in biofilms (118). Like for other adhesins, the N-terminal portion of Hwp1 is involved in its adhesive function; there is a central Ser/Thr-rich region subject to N and O glycosylation (Fig. 2) and a C-terminal signal for GPI anchor attachment (113). Hwp1 lacking the C-terminal 26 residues is found in the supernatant, consistent with loss of GPI anchor attachment (113).

Hwp1 is unique among adhesins analyzed to date in that it becomes covalently attached to mammalian cells: surface-exposed Hwp1 is a substrate for mammalian transglutaminases, which covalently attach Hwp1 to as-yet-unidentified proteins on the host cell surface (114). The N-terminal portion of Hwp1 is predicted to have a coiled-coil structure similar to mammalian small proline-rich proteins, which are natural substrates for mammalian transglutaminases (3, 113, 115, 116). Though *hwp1Δ/hwp1Δ* mutants still adhere to BECs under typical assay conditions, Hwp1 is required for *C. albicans* to adhere stably to BECs under more stringent conditions (114). Accordingly, loss of Hwp1 or addition of transglutaminase inhibitors results in reduced adherence when assayed under conditions with heat and detergents, which disrupt noncovalent attachments between cells (114).

The functional relevance of Hwp1-mediated adherence has been shown in several models of infection. Hwp1 is required for virulence in murine models of oropharyngeal and esophageal infection (119). These models use genetically immunocompromised mice, which are susceptible to *Candida* infections through invasion of tongue tissue, esophageal

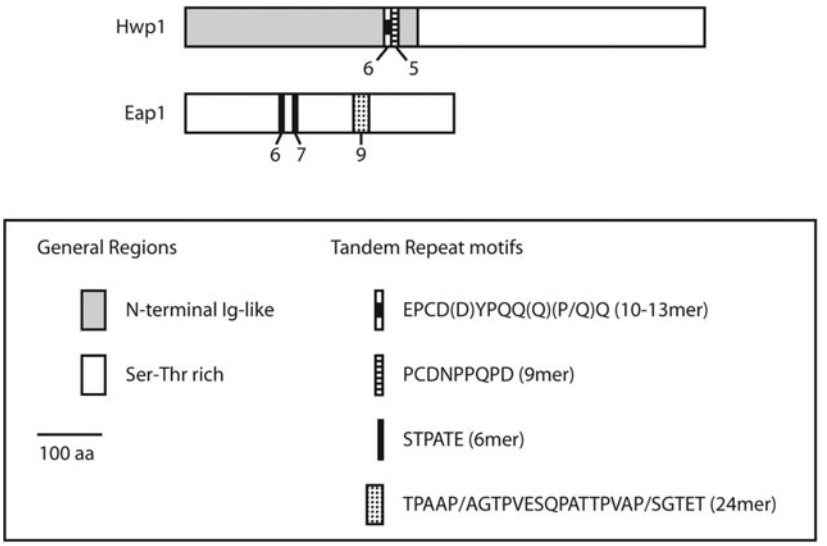

FIGURE 2 Schematic of protein domains in the *C. albicans* adhesins Hwp1 and Eap1. Protein domains and repeat motifs described in the section "Non-Als adhesins in *C. albicans*" are indicated in the key. Each drawing is to scale, with tandem repeats collapsed such that one motif unit is shown and the number of repeat units is indicated below the drawing. Motifs found in Hwp1 (115), as well as the motifs found in Eap1 (67, 70), have been described previously, though the number of tandem repeats was determined from our bioinformatic analysis and may differ from previously published analyses. Genes are as follows: *HWP1* (orf19.1321) and *EAP1* (orf19.1401). 10.1128/9781555817176.ch16f2

blockage, and tissue damage. Mice are infected superficially; observed mortality is thought to be due to severe mucosal damage and not due to disseminated candidiasis. A strain lacking *HWP1* had reduced invasion of tongue tissue, caused no damage to esophageal tissue, and was highly attenuated in causing host death (119). However, the *hwp1*Δ/*hwp1*Δ strain was not attenuated in endothelial cell damage assays or mouse models of disseminated infection (103); earlier studies had indicated that *HWP1* may have a role in disseminated infection, but these results are most likely due to the genomic position of the *URA3* auxotrophic marker (103,114, 119, 120).

Hwp1 also contributes to adhesion in the absence of mammalian transglutaminases, as shown by its role in biofilm formation in vitro (25, 84). In *C. albicans* biofilms, Hwp1 partners with Awp1 or Awp3 in order to mediate cell-cell adhesion (see discussion below) (87).

HWP1 transcription is highly upregulated in hyphal cells, and Hwp1 protein is detectable on the surface of germ tubes and true hyphae, but not yeast form cells (17, 115). Developmental regulation of *HWP1* is controlled by the transcription factor Efg1, which binds the *HWP1* promoter directly and activates expression of *HWP1* (7). *HWP1* mRNA is found in primary clinical samples from patients with oral and vaginal candidiasis, consistent with a role in colonization of those sites (81).

Additionally, *HWP1* is upregulated in mating-competent opaque **a/a** and α/α cells in *C. albicans* in response to mating pheromone. The presence of Hwp1 and two related proteins, Hwp2 and Rbt1, contributes to efficient mating between *C. albicans* cells (25). Hwp1, Hwp2, and Rbt1 proteins may help compatible mating partners adhere to one another. That said, mating efficiencies in these mutant strains were similar on liquid and solid media, suggesting that these proteins may not behave as classical agglutinins, which are expected to be particularly critical for adherence when mating occurs in liquid.

EAP1. Eap1 is a GPI-anchored protein that, like other adhesins, has an N-terminal signal sequence, a ligand-binding domain, a Ser/Thr-rich region, and a C-terminal ω site for GPI anchor attachment (Fig. 2) (68, 70). The two alleles of *EAP1* in *C. albicans* SC5314 differ strongly in the number of tandem repeats in the Ser/Thr-rich portion of the protein (~90 versus ~17 copies) (67). Eap1 also contains a second set of ~12 tandem repeats located C terminally to those previously mentioned. Systematic deletion of different protein domains of Eap1 indicated that repeated regions serve to project the N-terminal portion of Eap1 onto the cell surface (67). Northern blots examining *EAP1* expression in *C. albicans* found transcripts under both yeast- and hypha-inducing conditions, but expression under either of these conditions required Efg1 (68).

EAP1 was originally identified in a heterologous expression system examining *C. albicans* genes whose expression enables *S. cerevisiae* to adhere to polystyrene. *EAP1* can also functionally complement loss of the flocculin *FLO11* in *S. cerevisiae*, enabling pseudohyphal growth in diploids and haploid invasive growth in *flo11*Δ mutants (68). Consistent with a role for Eap1 in adherence under shear conditions, *eap1*Δ/*eap1*Δ cells had a 50% decrease in the number of cells adherent to polystyrene under flow conditions compared to wild-type cells. (70). Heterologous expression of *EAP1* also allows *S. cerevisiae* to adhere to HEK293 cultured epithelial cells (68, 69). Eap1 was found to contribute to *C. albicans* cell binding to epithelial cells using the flow cell system, since *eap1*Δ/*eap1*Δ cells showed a 30% reduction in binding relative to the wild type (70). Domain analysis of Eap1 expressed in *S. cerevisiae* shows a distinct modularity in the function of Eap1. While the N-terminal tandem repeat re-

gion mediates binding to HEK293 cells, the two regions of tandem repeats within Eap1 are clearly sufficient to mediate adhesion to polystyrene (67). The domain N-terminal to the tandem repeats plays no apparent role in polystyrene adherence, and the tandem repeat domains are neither necessary nor sufficient for adherence to HEK293 cells. This is perhaps the clearest example for any adhesin of multifunctionality achieved by the combination of different domains.

EAP1 also contributes to cell-cell adhesion between yeast cells. *EAP1* expressed in *S. cerevisiae* cells lacking their native flocculins enabled the *S. cerevisiae* cells to bind to a layer of *C. albicans* cells in a flow chamber (70). The ligand to which Eap1 binds on the surface of *C. albicans* cells is not known (70), but analysis of truncated versions of Eap1 has shown that the N-terminal portion of Eap1 is responsible for cell-cell adhesion. When expressed in *S. cerevisiae*, versions of Eap1 lacking the surface-expressed N-terminal domain are defective in haploid invasive growth assays and are unable to form cell aggregates in liquid media, two processes which require cell-cell adhesion (67). Notably, and consistent with a role in cell-cell interaction, haploid invasive growth was restored in *S. cerevisiae flo8*Δ cells by surface-expressed Eap1 N-terminal domain.

Expression and Function of *C. albicans* Adhesins in Biofilms

Biofilms are communities of microbes growing on a solid surface. In early biofilm development, cells adhere to a solid substrate, and subsequent growth depends on cell-to-cell adhesion and secretion of extracellular matrix. *C. albicans* biofilms are a mixture of yeast and hyphal cells, with yeast form cells forming the base layer in contact with the substrate and the hyphal cells forming the majority of the upper layers of the biofilm in contact with the environment. Adhesins play critical roles in all stages of biofilm growth, as they are necessary both for adherence to the substrate and for intracellular adhesion between neighboring yeasts in a more mature biofilm.

The transcriptional profile of cells grown in biofilms has been compared to that of planktonic cells in numerous studies. Microarray and reverse transcriptase quantitative PCR (RTqPCR) analyses have been employed to identify genes whose expression is regulated by growth in biofilms. Multiple studies have shown that *ALS1*, *ALS3*, and *HWP1* expression is upregulated in biofilms grown on abiotic surfaces and RHE models (12, 34, 37, 82, 83, 88, 131). RTqPCR studies found that *ALS2*, *ALS4*, and *ALS5* were also overexpressed in all biofilm models tested in the study (82). Expression of *ALS6* and *ALS9* was upregulated in some models of biofilms and downregulated in others, hinting at more complex regulation of these particular adhesins (82).

The role of Als and other adhesins in biofilm development has been investigated, initially in analyzing targets of Bcr1. *BCR1* encodes a transcription factor absolutely required for biofilm formation but, importantly, not for hyphal growth (85). Microarray analysis indicated that Bcr1 functions downstream of morphogenesis regulators specifically to regulate cell surface proteins and promote adhesion of *C. albicans*, without promoting a yeast-to-hypha transition (84, 85). Comparison of transcriptional profiles of planktonic *bcr1*Δ/*bcr1*Δ and complemented *bcr1*Δ/*bcr1*Δ + *pBCR1* strains grown under biofilm-inducing conditions identified a number of adhesins whose expression was altered in *bcr1*Δ/*bcr1*Δ mutants. In particular, *ALS3* expression was reduced 16-fold in *bcr1*Δ/*bcr1*Δ strains grown under these conditions. Expression levels of *ALS1* and *ALS9* were also decreased twofold in the *bcr1*Δ/*bcr1*Δ mutant, and expression of *ALS2*, *ALS4*, *ALS5*, and *ALS6* was not altered in the *bcr1*Δ/*bcr1*Δ strain (85).

In particular, Bcr1-mediated induction of *ALS3* is critical to promote biofilm growth. Strains lacking either *BCR1* or *ALS3* are unable to form biofilms in vitro (84, 85), but heterologous expression of *ALS3* can fully rescue biofilm growth of *bcr1*Δ/*bcr1*Δ strains in vitro and in vivo (84). The adhesins Als1 and Hwp1 are also regulated by Bcr1 and contribute to biofilm formation; however, loss of these genes individually does not result in the extreme loss of biofilm growth observed in *bcr1*Δ/*bcr1*Δ or *als3*Δ/*als3*Δ mutants. Consistent with some role for these adhesins, heterologous expression of *ALS1* and *HWP1* in *bcr1*Δ/*bcr1*Δ mutants only partially rescues the biofilm growth defect (84). In contrast to the absolute requirement for *ALS3* for in vitro biofilm formation, *als3*Δ/*als3*Δ mutants were capable of forming biofilms in vivo, in venous catheter models (84). These results may indicate that in the context of the in vivo catheter models, other adhesins regulated by Bcr1 are able to compensate for the loss of *ALS3*. As mentioned above, *HWP1* is a target of Bcr1 regulation. Strains lacking *HWP1* form biofilms with a threefold reduction in biomass compared to that of biofilms of wild-type cells. The *hwp1*Δ/*hwp1*Δ biofilms have few hyphae, and both yeast and hyphal cells are apparent in the supernatant, indicating that Hwp1 may have a role in retaining cells in the biofilm structure (86). Hwp1 also contributes to biofilm growth in vivo in a rat venous catheter model, with *hwp1*Δ/*hwp1*Δ mutants being highly defective in biofilm formation (86).

These findings prompted further examination of the adhesive roles of Als1, Als3, and Hwp1 in biofilm formation, particularly in vivo. With the knowledge that *ALS1* and *ALS3* are similar in sequence, the possibility that Als1 and Als3 have overlapping function in biofilms was tested. Though both *als1*Δ/*als1*Δ or *als3*Δ/*als3*Δ mutants are able to form mature biofilms in vivo, the *als1*Δ/*als1*Δ *als3*Δ/*als3*Δ double mutant is severely defective; no adherent cells were observed on the catheter lumen (87). Introducing one allele of either *ALS1* or *ALS3* results in occasional adherent cells but not biofilm formation in the in vivo model. Together, these data indicate that *ALS1* and *ALS3* are functionally redundant and that two alleles of *ALS1* or *ALS3* are needed to form a biofilm in vivo. Importantly, ectopic expression of *HWP1* was unable to rescue biofilm formation in the *als1*Δ/*als1*Δ *als3*Δ/*als3*Δ strains, perhaps indicating that Hwp1 requires Als1 or Als3 for function during biofilm growth. Consistent with this notion, a mixture of *hwp1*Δ/*hwp1*Δ and *als1*Δ/*als1*Δ *als3*Δ/*als3*Δ cells was able to form confluent biofilms both in vitro and in vivo (87). The ability of the strain mixture to grow into biofilms suggested that these three adhesins function together, with Hwp1 being a binding partner for either Als1 or Als3. Consistent with this, heterologous expression of *HWP1* in *S. cerevisiae* enabled binding to *C. albicans* hyphal cells, which typically express abundant Als1 and Als3. This Hwp1-mediated binding decreased twofold when tested with *als1*Δ/*als1*Δ *als3*Δ/*als3*Δ *Candida* cells. Together, these results suggest that Hwp1 mediates binding to *C. albicans* directly and that this adherence is strengthened by the presence of Als1 and Als3. A potential role for other Als adhesins in biofilm formation may be indicated, since overexpression of a subset of *ALS* family members can also rescue biofilm formation in *als1*Δ/*als1*Δ *als3*Δ/*als3*Δ mutant background in vitro. However, in vivo biofilm formation was rescued by overexpression of only a subset of *ALS* genes (87).

EAP1 also contributes to biofilm formation in *C. albicans*. Strains lacking *EAP1* were able to form rudimentary biofilms consisting of a few layers of yeast cells bound to the substrate in a flow chamber. These biofilms proved to be unstable and were washed away as time progressed, likely due to increased effective shear forces on the cells as they grew (70). The *eap1*Δ/*eap1*Δ mutants were also unable to form biofilms in vivo in rat catheter models of biofilm formation (70). Quantitative RT-PCR studies showed that *EAP1* expression is about twofold higher in cells grown as biofilms in comparison with planktonically grown cells. This defect in biofilm growth likely reflects the combined defects in substrate adhesion and cell-cell adhesion exhibited by *eap1*Δ/*eap1*Δ strains.

Adhesins in *Candida glabrata*

The overall structure of the cell wall in *C. glabrata* is similar to that of *C. albicans*, consisting of a network of β-1,3-glucan, β-1,6-glucan, chitin, and mannoproteins. Strikingly, proteins account for 6% of the dry weight of the cell wall in *C. glabrata*, which is roughly a 50% increase in protein content over the cell wall of *C. albicans* or *S. cerevisiae* (21).

Global in silico analysis of the *C. glabrata* genomic sequence of strain ATCC 2001 (CBS138) identified putative adhesins by searching for proteins with an N-terminal effector domain, followed by a low-complexity domain with tandem repeats and a high proportion of Ser and Thr residues, which are hallmarks of adhesins (21, 128). Based on the comprehensive annotation of the *C. glabrata* genome released in 2004, 67 putative adhesin-like GPI-anchored proteins were noted, though functional analysis for most of these has not been carried out (21). The putative adhesins can be classified into seven protein families, and the best-studied family is the Epa adhesin family, with 17 members identifiable in the 2004 annotation of the *C. glabrata* genome of strain CBS138 (Table 1). In another common laboratory strain of *C. glabrata*, BG2, 23 *Epa* genes have been identified to date (57). The discrepancy between the presence of Epa family members in these two strains could represent real differences between the strains or incomplete sequencing and annotation of either strain due to difficulties of sequencing DNA near the telomeres.

In preparation for protein domain analysis for this chapter, sequence of *C. glabrata* CBS138 was obtained from Genolevures in August 2010 (106), and it was found that systematic identifications (IDs) of *EPA9*, *EPA10*, *EPA19*, and *EPA22* have changed from previous versions. Assignment of current systematic IDs (Table 1) was confirmed by matching the 5′ portion of the gene to the previously defined open reading frame (ORF). Assignment of *EPA19* in CBS138 has been removed, but BLAST analysis places the ATG of *EPA19* 4,309 bp from the end of Chr A-L (complement strand), and current sequence assembly shows this ORF split over two reading frames. Sequences retrieved with the systematic ID for *EPA7* (in CBS138) and *EPA21* contain internal stop codons; subsequent analysis of these genes (for Color Plate 6) only considers sequence up to the first stop codon.

Structure/Function Analysis of Epa Adhesins

Like the adhesins in *C. albicans* described above, adhesins in *C. glabrata* have a modular domain structure (Color Plate 6). They contain a signal sequence, a ligand-binding domain, a Ser/Thr-rich stalk often containing tan-

TABLE 1 *EPA* genes in *C. glabrata*[a]

	CBS138				
Gene	Old systematic ID	Current systematic ID	BG2 GenBank ID	Chr	Arm
EPA1	CAGL0E06644g	CAGL0E06644g	AY344226	E	R
EPA2	CAGL0E06666g	CAGL0E06666g	AY344226	E	R
EPA3	CAGL0E06688g	CAGL0E06688g	AY344226	E	R
EPA4	NA	NA	AY344225	I	R
EPA5	NA	NA	AY344225	I	R
EPA6	CAGL0C00110g	CAGL0C00110g	AY646925	C	L
EPA7	CAGL0C05643g	CAGL0C05643g	AY646926	C	R
EPA8	CAGL0C00847g	CAGL0C00847g		C	L
EPA9	CAGL0A01386g	CAGL0A01366g		A	R
EPA10	CAGL0A01265g	CAGL0A01284g		A	R
EPA11	CAGL0L13310g	CAGL0L13299g		L	R
EPA12	CAGL0M00132g	CAGL0M00132g		M	L
EPA13	CAGL0L13332g	CAGL0L13332g		L	R
EPA15	CAGL0J11968g	CAGL0J11968g		J	R
EPA19	CAGL0A00110g/00121g	No assignment		A	L
EPA20	CAGL0E00275g	CAGL0E00275g		E	L
EPA21	CAGL0D06732g/06754g	CAGL0D06732g		D	R
EPA22	CAGL0K00132g/00143g	CAGL0K00170g		K	L
EPA23	CAGL0I00220g	CAGL0I00220g		I	L

[a]"Old systematic ID" refers to the designations specified in the original 2004 annotation of *C. glabrata* strain CBS138. "Current systematic ID" specifies the systematic IDs as of August 2010. In strain BG2, *EPA1* to *EPA7* have been sequenced and are publicly available on GenBank. The table also lists the chromosome arm on which each *EPA* is located in strain CBS138, except for *EPA4* and *EPA5* which are only found in BG2. NA, not applicable.

demly repeated sequences, and a GPI anchor for attachment to the cell well. Many protein domains shown in Color Plate 6 were defined in sequence analysis performed for this chapter and may differ from previously published analyses due to strain differences, sequence source, sequence definitions of the repeat units, or number of tandem repeats. Protein sequence was derived from the publicly available sequence for CBS138 (Genolevures, August 2010 [106]) and BG2 (11, 22). New repeat motifs were identified manually and using online software (RePro [43]), and the consensus (75% identity) is specified in the legend for Color Plate 6.

All Epa sequences obtained contained a signal sequence for their secretion at their N termini (not shown in Color Plate 6). Following the signal sequence, the N-terminal portions of the proteins function as the ligand-binding domain; this function been shown for certain Epa proteins, where expression of the N-terminal ~300 amino acids in *S. cerevisiae* mediates ligand binding (31, 140). Though the length of this ligand-binding domain is generally conserved, the amino acid sequences are not necessarily related between family members. The ligand-binding domain is followed by a region of tandem repeats containing a large proportion of Ser and Thr residues which serve as sites for glycosylation. Epa family members contain a wide variety of repeated sequences of different lengths (Color Plate 6), and *EPA* genes may be subclassified based on the types of repeat motifs present. For instance, *EPA2*, *EPA3*, *EPA4*, *EPA5*, and *EPA22* are highly related in sharing variable numbers of two distinct repeat motifs. Note that the number and organization of the repeats for Epa2 and Epa3 differ between the CBS138 and BG2 strains. The VSHITT tandem repeat sequence was found in almost half of the predicted adhesins in *C. glabrata*, in all subclasses of adhesive proteins (21). The VSHITT domain has also been identified as SHITT megasatellite domains in a separate bioinformatics analysis (121). Other internal domains defined in *C. albicans* Als adhesins, such as the amyloid-forming T region, have not been formally identified or analyzed in *C. glabrata* to date.

A C-terminal GPI anchor signal is required for adhesin function (31). The sequences for most of the Epa proteins in Color Plate 6 contain hydrophobic sequences for attachment of GPI anchors at their C termini, the exceptions being Epa7, Epa11, and Epa21. The Epa7 protein sequence in CBS138 appears to contain an internal stop codon, likely rendering the N-terminal fragment shown in Color Plate 6 nonfunctional, as it would lack a GPI anchor. The annotated Epa11 protein sequence does not contain a C-terminal hydrophobic sequence (assessed with Kyte and Doolittle hydrophobicity profiles in BioEdit software), perhaps indicating that the sequence is incorrectly annotated or assembled. The sequence for *EPA21* lies at the extreme end of the Chr D sequence and likely represents a partial sequence, as it is much shorter than other Epas and contains a partial VSHITT domain.

Epa Adhesin Roles in Epithelial and Endothelial Adherence and Infection Models

Several models of infection have been employed to determine the potential contribution of individual *EPA* genes to adherence of *C. glabrata*. Epa1 mediates adherence to epithelial cells, and *epa1*Δ mutants show a 95% reduction in adherence (15). The Epa adhesins characterized to date are Ca^{2+}-dependent lectins that mediate adherence by binding host carbohydrate (15, 140). Cells expressing Epa1 can bind a number of mammalian epithelial cell lines, exhibiting the most efficient binding to lec2 cells, which have a defect in sialic acid modification of surface glycans and expose elevated levels of terminal *N*-acetyllactosamine (Galβ1-4GlcNAc). Yeast binding to lec1 and lec8 cells, which have reduced levels of Gal or GlcNAc on their surfaces, is reduced to background levels (140). *C. glabrata* cells are also able to bind to cultured uroepithelial cells. Binding to any epithelial cell tested to date can be mediated by the adhesins Epa1, Epa6, and Epa7 (24). By contrast, adherence to endothelial cells is apparently mediated by Epa1 and Epa7 but not Epa6 (140).

In disseminated models of infection, *epa1*Δ mutants show no phenotype (15). An *epa(1-5)*Δ mutant showed a modest decrease in colonization of kidney in a disseminated model of infection (22). An *epa(1,6,7)*Δ mutant strain, with deletions of the three major adhesins identified to date, shows a decreased ability to colonize the bladder in mouse models of urinary tract infection (24).

Binding Specificities of Epa Proteins

Glycan microarrays have been employed to analyze the carbohydrate binding specificities of Epa1, Epa6, and Epa7 (140). The N-terminal ligand-binding domain of each Epa protein was expressed on the surface of *S. cerevisiae* cells and assayed for binding to immobilized carbohydrates. It was found that all three adhesins bound highly related carbohydrates. All had an absolute requirement for a terminal galactose residue. Epa6 has the broadest range of ligands, binding essentially all ligands with a terminal galactose, including α-1,4, α-1,3, β-1,4, or β-1,3 linkages to glucose (Glc) or GlcNac. Epa1 and Epa7 were more limited in their binding specificities, being unable to bind the α-linked subset of glycans. Because Epa6 and Epa7 are more than 90% similar at the protein level, these differences in binding specificity motivated domain-swapping experiments, which identified five residues in the N termini of these proteins which are largely responsible for the respective specificities (140) and which likely constitute the ligand binding cleft of the protein. The binding specificity for the other putative Epa adhesins has not been reported.

Regulation of *C. glabrata* Adhesin Expression

Notably, 44 of the 67 predicted adhesins in *C. glabrata* are encoded near telomeres (21), and 14 of the 17 *EPA* genes in CBS138 are located subtelomerically (Fig. 3). This position leaves them subject to subtelomeric transcriptional silencing. The silencing of the *EPA* loci requires the NAD^+-dependent histone deacetylase (HDAC) Sir2 and associated proteins Sir3 and Sir4 (22, 24, 52), as well as proteins involved in controlling telomere length—Rif1, Ku70, and Ku80 (11, 101)—and Rap1, which binds the telomeric repeats and is thought to recruit Sir2 to the telomeres (22).

When *C. glabrata* is grown under standard laboratory conditions, the *EPA* genes are not typically expressed due to telomeric silencing. Effective subtelomeric silencing is illustrated by the fact that a *URA3* gene inserted subtelomerically is sufficiently repressed to allow cells to grow on media containing 5-fluoroorotic acid (22). Disruption of the silencing machinery relieves subtelomeric silencing, and *EPA* genes are derepressed (11). However, regulation of *EPA* gene expression is complex, and expression levels of individual *EPA* genes depend on additional factors beyond Sir-mediated silencing. Thus, mutation of key silencing proteins does not dramatically increase expression of all the *EPA* genes. There is also a requirement for additional positive transcriptional activators in order to transcribe *EPA* loci; for example, *FLO8* and *MSS11*, encoding two transcriptional

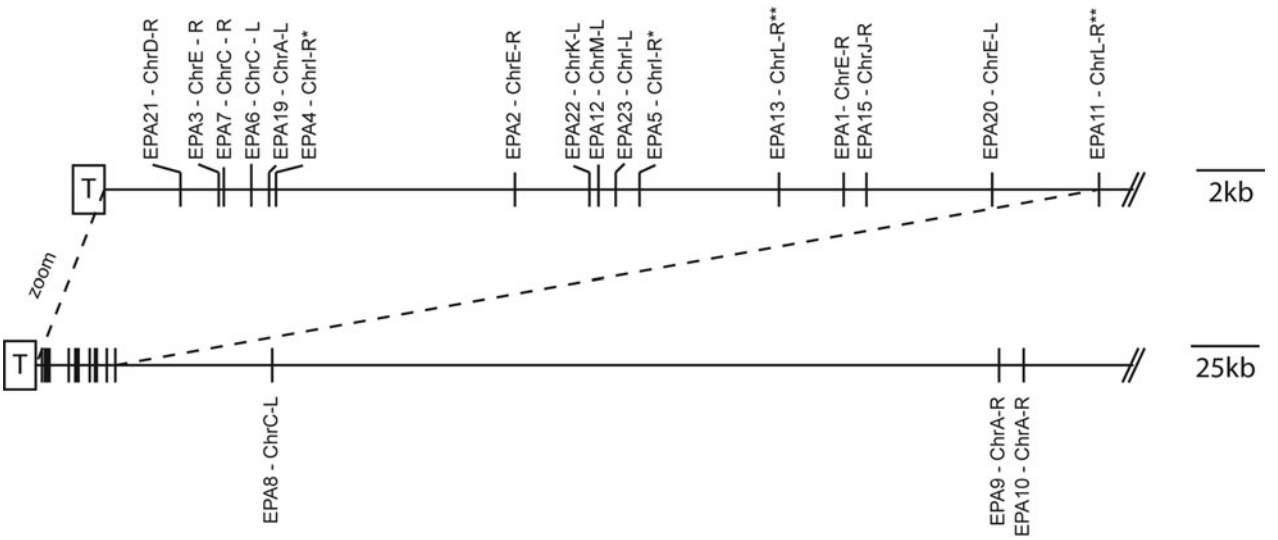

FIGURE 3 Diagram of *EPA* locations with respect to chromosome ends. Shown is a collapsed view of *EPA* positioning with respect to chromosome ends. The boxed T designates the telomere. *, *EPA4* and *EPA5* are present in the BG2 strain of *C. glabrata* but not CBS138. **, the published sequence of Chr L-R stops in the ribosomal DNA locus, which lies between *EPA13* and the telomere. Thus, the distances mapped for *EPA11* and *EPA13* are likely underestimates, as it is unknown how many ribosomal DNA repeats lie between the *EPA*s and the telomere on this chromosome. 10.1128/9781555817176.ch16f3

activators, are required for transcription of some *EPA* genes (80). In addition, loss of certain silencing components does not alter silencing of all subtelomeric *EPA* loci equally. For instance, loss of Ku70/Ku80 derepresses *EPA6* and *EPA7* (located at Chr C-L and Chr C-R, respectively), but *EPA1*, *EPA2*, and *EPA3* (all located at Chr E-R) expression is not increased in these silencing mutants (101). Loss of Rif1 derepresses the *EPA6* but not the *EPA7* locus. Some of these regulatory differences may be due to the presence of *cis*-acting silencers, such as the silencer found near the *EPA1* cluster, which may promote the binding of silencing proteins far from the telomeres (101). Subtelomeric silencing likely also controls expression of other predicted adhesins in *C. glabrata*, as 44 of the predicted 67 adhesins are located subtelomerically, and putative adhesins are located on every end of every chromosome (21).

EPA genes in *C. glabrata* are not typically expressed during log-phase laboratory growth, though *EPA1* is transiently expressed in early exponential phase (15). However, expression of several *EPA* genes, notably *EPA1*, *EPA6*, and *EPA7*, can be induced in response to environmental conditions. The silencing protein Sir2 is a NAD^+-dependent HDAC, and because *C. glabrata* is a NAD^+ auxotroph, cells must obtain NAD^+ precursors from the environment (77). Limiting levels of precursors in the environment lead to low NAD^+ levels in the cells and loss of silencing (76). For instance, *EPA6* expression is induced when nicotinic acid (NA) or other NAD^+ precursors are limiting in the media (24). The NA responsiveness of *EPA* expression has functional consequences: *C. glabrata* grown in urine (but not in urine supplemented with NA) displays increased adherence to epithelial cells, due to increased expression of *EPA1*, *EPA6*, and *EPA7*. *EPA6* expression is also induced during mouse models of urinary tract infections, and *epa(1,6,7)*Δ strains are attenuated for virulence in a mouse model of urinary tract infection (24).

Similar to adhesins in *C. albicans*, growth in biofilms induces adhesin expression in *C. glabrata*. Specifically, *EPA6* and *EPA7* are upregulated in biofilms, compared to planktonic cells (52). Regulation of *EPA6* and *EPA7* in biofilms occurs through the kinase Yak1, though the target of Yak1 is not currently known. The biofilm growth defect in *epa6*Δ mutants seems due to impaired yeast-yeast adhesion, since the *epa6*Δ strains appear unaltered in initial adherence to inert substrates but are compromised in subsequent higher-level biofilm formation (52). How this role of *EPA6* meshes with the glycan specificity (described above) is not clear, and this cell-cell interaction role of *EPA6* may depend on other domains than the N-terminal lectin domain.

EPA6 expression is also induced in response to the combination of low oxygen and the chemical preservatives parabens and sorbic acid (80). *EPA6* induction in response to preservatives is independent of the orthologues of the *S. cerevisiae* stress response transcription factors Msn2 and Msn4 and also independent of War1, a transcription factor involved in the weak-acid response (80). Induction of *EPA6* under these conditions instead depended on orthologues of the *S. cerevisiae* Flo8 and Mss11 transcription factors (80). Interestingly, in *S. cerevisiae*, the transcription factors Flo8 and Mss11 regulate expression of the *FLO* genes (see discussion below), indicating a conservation of regulation between *S. cerevisiae* and *C. glabrata*.

Putative Adhesin Families in *C. glabrata*

While the *EPA* family is the largest and best-studied family of adhesins in *C. glabrata*, the genome is predicted to encode additional adhesive proteins. In silico analyses of the CBS138 genome sought to assemble a comprehensive list of putative adhesins (21, 128). Proteins with a modular domain structure, including an N-terminal globular domain followed by a low-complexity, Ser/Thr-rich domain containing tandem repeats, were considered. In some cases,

genes were apparently fractured into multiple adjacent pieces, likely reflecting technical difficulties sequencing subtelomeric regions rather than the true structure of these coding regions. When fractured ORFs were considered, a total of 67 adhesin-like proteins were identified in the CBS138 genome (21).

In addition to the family of 17 *EPA* genes identified (family I), six other families of putative adhesins were defined. The *PWP* family (II) contains seven members related by the presence of a PA14 domain in their ligand-binding domain. The PA14 domain, also found in the proteins corresponding to the *EPA* genes as well as in the *FLO* genes in *S. cerevisiae*, is a carbohydrate binding module present in a large range of sugar-binding proteins in eukaryotes and prokaryotes (98). Two adhesins in family IV have similarity to the protein corresponding to *DAN1* in *S. cerevisiae*, another subtelomeric adhesin whose expression is regulated by oxygen availability in the environment (95). The putative adhesins classified into other families do not have striking homology to known adhesins in *C. glabrata* or other yeasts, and their functions have not been reported.

Mass spectrometry was also used to identify covalently linked CWPs, including adhesins, on the surface of *C. glabrata*. In total, five adhesins were detected: Epa6 and four uncharacterized proteins that were subsequently named Awp1 to -4 (adhesin-like wall protein). Mass spectrometry was performed on two strains—ATCC 90876 (clinical isolate) and ATCC 2001 (sequenced clinical isolate CBS138)—grown to log or stationary phase at either 30 or 37°C (21). The Awps are members of two larger families of putative adhesins (Awp1 and Awp3 from family VI and Awp2 and Awp4 from family V). Awp2 and Awp4 contain VSHITT megasatellite sequences in their Ser/Thr-rich portions. This motif has been found in 31 of 67 adhesin-like proteins, across six of the seven adhesin-like families, including the Epas (21, 121).

Thus, a handful of the putative adhesins predicted by bioinformatics have also been shown to be expressed on the cell surface. Additional work will need to confirm and characterize the adhesive properties of the Awp proteins and other putative adhesins identified bioinformatically.

Flo Adhesins in *S. cerevisiae*

The *S. cerevisiae* genome encodes a family of five adhesins, Flo1, Flo5, Flo9, Flo10, and Flo11. Expression of these adhesins is activated by the transcription factor Flo8. The Flo proteins have the general structure of adhesins described above: an N-terminal signal sequence is followed by a lectin-like binding domain, a Ser/Thr-rich repeat domain, and a signal for GPI anchor attachment at the C terminus.

All of the *FLO* genes, with the exception of *FLO11*, are located near telomeres in *S. cerevisiae*. A combination of epigenetic and promoter-specific regulation results in *FLO1*, *FLO5*, *FLO9*, and *FLO10* being transcriptionally silent in Σ1278b strain. *FLO11* expression is regulated in response to environmental conditions: nitrogen starvation conditions activate the mitogen-activated protein kinase (MAPK) and PKA signaling cascades, which together induce the expression of *FLO11* (102).

Adhesive Functions of Flo Proteins

When expressed, the Flo proteins contribute to a variety of behaviors that rely on adhesion. More specific interactions between adhesins lead to yeast-yeast adhesion during flocculation, a process by which yeast cells bind to each other and sediment out of a liquid growth environment in a calcium-dependent manner, which is particularly important industrially in fermentation, aiding in clarifying wine and beer. They also have roles in hydrophobic interactions with substrates as well as haploid invasive growth and pseudohyphal growth.

The *FLO* genes play essential roles during flocculation, a process by which yeast cells bind to the mannose residues on the surface of neighboring cells, forming clusters of cells called flocs, and sediment (62). Mechanisms of flocculation have been classified based on the sugars which inhibit floc formation. Flocculation can be mediated by Flo1, Flo5, Flo9, and Flo10, but not by Flo11 (36, 123). Flo1-type flocculation depends on binding to mannose sugars decorating glycosylated proteins on neighboring cells. This variety of flocculation is mediated primarily by Flo1 as well as the related proteins Flo5 and Flo9. As an indication of ligand specificity, Flo1 flocculation is inhibited only by high (0.8 to 1 M) concentrations of mannose (36, 123), while Flo5- and Flo9-mediated flocculation is sensitive to inhibition by mannose and, to a lesser extent, maltose and glucose (123). So-called NewFlo-type flocculation, mediated by Flo10, can be inhibited by mannose, glucose, sucrose, and maltose (41, 104). Consistent with the inability of *S. cerevisiae* cells to adhere to mammalian tissues, galactose, a sugar residue normally found on the glycosylated surfaces of mammalian cells, does not inhibit flocculation by any of the Flo proteins (123).

Cell surface hydrophobicity is increased by expression of *FLO* genes, as measured using a biphasic assay where cells in suspension partition into an aqueous layer or organic solvent (octane) (36, 123). Altered hydrophobicity is thought to promote yeast binding to hydrophobic surfaces, such as plastic (41, 54, 123), though overexpression of any *FLO* gene increased cell surface hydrophobicity, while only expression of *FLO5* or *FLO9* caused the cells to adhere to polystyrene plates (123).

FLO11 has a primary role in cell-cell interactions needed for invasive growth. Under stressful conditions, strains alter cell morphology and budding pattern to allow the formation of pseudohyphal chains of cells and to penetrate into the growth surface, such as agar media (8, 16, 99), probably to permit stressed cells to scavenge for the nutrients they lack. In diploid cells, *FLO11* is required for pseudohyphal growth in response to nitrogen starvation, and haploid strains that express *FLO10* or *FLO11* (but not *FLO1*, *FLO5*, or *FLO9*) are capable of haploid invasive growth (36, 123).

Strain-Specific Regulation of *FLO* Genes

The regulation of the *FLO* genes, which are responsive to nutritional status as well as growth phases, is complex. The laboratory strain S288C is generally nonadhesive due to a nonsense mutation in the transcriptional activator *FLO8*, effectively silencing *FLO1* and *FLO11* in this strain. Adhesive phenotypes may be rescued in S288C by restoring a functional copy of *FLO8* (5, 27, 33, 63, 72). In S288C, the remaining *FLO* genes (*FLO5*, *FLO9*, and *FLO10*) are typically silenced, even if Flo8 is functional and other transcriptional silencers are disrupted (27). Additionally, *FLO* genes are repressed by the Tup1/Ssn6 complex, the HDAC Hda1 and the global transcriptional cofactor Mediator (40, 133). *FLO10* and *FLO11* expression is regulated by Flo8 and Tup1/Ssn6 and, in addition, by the Tec1 and Ste12 transcription factors downstream of the MAPK pathway (42, 102).

In addition, *FLO10* and *FLO11* are strongly regulated by epigenetic regulation. In a laboratory strain of *S. cerevisiae*, Σ1278b, only *FLO11* is expressed (41); the other telomere-proximal *FLO* genes are silenced. In clonal populations of

Σ1278b, *FLO11* expression varies from cell to cell, due to a combination of promoter-specific and positional silencing. Though *FLO11* is not located subtelomerically (>40 kb), proper expression of *FLO11* still depends on its location within the genome, as the promoter is not properly silenced when moved from its normal locus (42). Epigenetic silencing of the *FLO11* promoter requires the transcription factor Sfl1, the corepressor Tup1, and the HDAC Hda1 (41, 42, 91). The telomeric silencing machinery—Ku70, Ku80, the Sir complex, and Hst1 to Hst4—is not required for silencing the *FLO11* promoter (42). Intriguingly, *FLO10*, which can also mediate invasive growth, when overexpressed contributes to haploid invasive growth in Σ1278b in strain variants in which *IRA1* or *IRA2*, which correspond to Ras GTPase-activating proteins, are inactive. Ira-deficient strains are capable of flocculation due to derepression of *FLO10* (42). Similar to *FLO11* regulation, *FLO10* expression is also epigenetically regulated in these strains. Expression of *FLO10* is regulated by the HDACs *HST1* and *HST2*, but not *SIR2*. These studies suggest an integration of PKA signaling and epigenetic regulation of flocculins (42). Possible mechanisms for switching between epigenetic states are suggested by an analysis of the *FLO11* promoter. There, two noncoding RNAs (ncRNAs), *PWR1* and *ICR1*, in the large promoter region are reciprocally regulated, probably by a mechanism of mutual transcriptional interference (10). These ncRNAs are regulated by the transcription factors Flo8 and Sfl1. The transcription of *ICR1* stably represses *PWR1* as well as interfering with transcription of *FLO11* itself; by contrast, transcription of *PWR1* prevents transcription of *ICR1* and allows *FLO11* transcription. This additional layer of complexity again points to the complex regulation of cell surface proteins in yeast and presages equally complex regulation in *Candida*.

EVOLUTION OF ADHESIN FAMILIES

A notable feature of fungal evolution is the change in copy number for given genes among species, with some functional classes of genes showing highly variable copy number across species (127). Notably, the adhesin genes of *S. cerevisiae* and *C. glabrata* are concentrated in subtelomeric regions of the genome (11, 21, 22; reviewed in reference 125). Evolution of adhesin families is likely strongly influenced by their subtelomeric location. In silico analyses of fungal gene families have found that families with members located at subtelomeres show a tendency towards rapid expansion and divergence of the family members, in comparison to families with genes located elsewhere on the chromosome (9). This may be due to increased recombination rates in the subtelomeric regions, which could allow recombination between repetitive sequences in the genes found there, such as the 108-bp TRDs in the *ALS* genes or 135-bp repeats in the *FLO* genes. Enhanced recombination rates at the subtelomeres may be tolerated better by the yeast, because subtelomeric genes are often epigenetically silenced and are not typically involved in housekeeping functions of the cell (9), and genomic rearrangement may be less likely to disrupt essential functions of the cell. Expansion of the gene family may facilitate functional divergence, since selection pressures may be relieved on duplicate copies of a given gene. As duplicated adhesins diverge, each copy may evolve a specific regulatory program as well as specialized ligand recognition, thereby broadening the adhesive capabilities of the yeast.

The structure of the yeast adhesin itself also has an important effect on generation of adhesin diversity. The central Ser/Thr-rich region of *Candida* and *S. cerevisiae* adhesins very often includes a series of tandem repeats. A common feature of some of these repeats is that they are highly conserved at both the amino acid and nucleotide levels. This nucleotide conservation suggests that these could be substrates for homologous recombination (reviewed in reference 126). Unequal crossing over between the two alleles of a single adhesin gene would create adhesin alleles with different numbers of tandem repeat units than either of the original copies. The repeat number can likewise also change due to slippage during DNA synthesis. Recombination between tandem repeats shared between different adhesin genes could result in new combinations of N-terminal ligand-binding domains and C-terminal domains. This shuffling of functional domains could in principle generate tremendous functional diversity within the population. Additionally, such recombination could in principle move adhesin genes away from or closer to *cis*-acting control regions that would affect transcriptional expression.

The best evidence showing how recombination generates diversity comes from analysis of the *FLO* genes in *S. cerevisiae*. There, tandem repeats within a gene were shown to be substrates for elevated levels of homologous recombination, and recombinationally generated length variants of *FLO1* were shown to result in functional differences in adherence to plastic and in flocculation (28, 124). Notably, in the evolution of "flor" strains of *S. cerevisiae*, used to make sherry, an increase in tandem repeat copy number in the *FLO11* gene is partly responsible for the increased hydrophobicity of these strains and their ability to form a characteristic buoyant floating biofilm in fermentation (28, 124). In *C. glabrata*, some copy number variation in the tandem repeats in *EPA* genes has been noted (28, 124), and the tandem repeats of the majority of the *EPA* genes are highly conserved repeats at the nucleotide level, consistent with their being substrates for recombination. *Candida glabrata* also contains two major classes of megasatellites, which are repeat sequences with a motif size between 135 and 300 bp, and are not found in other hemiascomycete yeast species. The SHITT megasatellite consists of a 135-bp repeat and is found in 11 genes and 3 pseudogenes. The SFFIT megasatellite has a 300-bp repeat motif and is located in 12 genes and 6 pseudogenes. These megasatellites tend to be found in subtelomeric genes (122). In silico clustering analysis concluded that the megasatellites may propagate by duplication of the gene within which they reside, as well as through gene conversion events and shuffling of DNA motifs between megasatellites on different chromosomes (100).

Recombination would be predicted not only to change tandem repeat copy number but also to shuffle N and C termini of adhesins sharing the same tandem repeat region. In *C. albicans*, *ALS5* may be an example of such a "hybrid" adhesin. The 5′ portion of *ALS5* DNA sequence encoding the N-terminal ligand-binding domain is most closely related to *ALS1* and *ALS3* in the 5′ portion that encodes the N-terminal ligand-binding domain (46), while the *ALS5* 3′ sequence encoding the TRD and C-terminal domain is more conserved with *ALS6* (47). Analysis of *ALS* sequences points to the diversification of adhesin sequence, as a result of changes in repeat unit copy number within the TRD. The genes *ALS1*, *ALS5*, and *ALS9* are located adjacent to one another on chromosome 6. Each of these three genes is present as a long and a short version, which vary based on the size of the TRDs. Remarkably, the long versions of *ALS1*, *ALS5*, and *ALS9* are all carried on one copy of chromosome 6, and the corresponding short alleles are on the other copy

of chromosome 6 (139). *ALS3* alleles are also heterozygous with respect to the number of tandemly repeated units encoded on each homologous chromosome (89). The *ALS7* sequence was analyzed in 66 clinical isolates of *C. albicans*, and 21 different TRD lengths were identified, where a given allele could have between 1 and 33 repeats of the 108-bp repeat unit (132). These *ALS7* alleles combined to make 30 different genotypes (when only considering the TRD length of *ALS7*), illustrating that clinical isolates have a wide range of adhesin genotypes. Similar trends were seen when analyzing tandem repeat numbers present in *ALS5* and *ALS6* alleles among clinical isolates; however, it was also found that *ALS5* was absent in many clinical isolates due to recombination events between direct repeat sequences flanking the *ALS5* gene (137). A broad, comprehensive analysis of tandem repeat number in all of the *ALS* genes across 43 isolates representing four clades showed striking diversity in repeat number both between strains and, in many cases, between alleles within a strain. While this study found no association between repeat number for any given adhesin gene and virulence in a murine model of disseminated infection, it demonstrated clearly the extremely large degree of adhesin gene variation brought about by the unstable tandem repeat (78).

CONCLUDING REMARKS

CWPs are key to the interaction of fungi with the environment, and not surprisingly, changes within the CWP repertoire accompany the evolutionary adaptation to a particular niche. Closely related species like *C. glabrata* and *S. cerevisiae*, though highly related across the genome as a whole, possess very distinct sets of surface proteins adapted to their specific lifestyles. The relatively limited repertoire of *FLO* genes in *S. cerevisiae* primarily serves to mediate yeast-yeast interactions that adapt it to growth in communities/biofilms as well as to respond to nutritional cues. Notably, both *C. glabrata* and *C. albicans* have evolved large distinct families of adhesins and putative adhesins, which in principle confer a selective advantage in its adaptation to the environment of the host. While the environments to which *S. cerevisiae* and *Candida* have adapted are quite different, certain themes are conserved across the adhesin families. Subtle differences in adhesin substrate specificity serve to distinguish family members and point to specialization of function. Exploration of sequence variation among adhesin genes in multiple isolates of the same species points as well to the dramatic cell-to-cell variation and accompanying functional diversification in genes within these families. This variation is in some sense engineered. In *S. cerevisiae* and *C. glabrata*, telomeric location likely increases rates of recombination, and in all three species, hard-wired parts of gene structure—namely, the presence of tandem repeats (of completely different sequence in different species) shared between different family members—appear to facilitate recombinational exchange between different genes and thereby drive evolutionary functional diversification. In fungal pathogens, understanding the function and regulation of individual members of these families not only will help define their individual role in fungal physiology but also will help define the selective pressures in host niches that drive fungal evolution.

REFERENCES

1. **Almeida, R. S., S. Brunke, A. Albrecht, S. Thewes, M. Laue, J. E. Edwards, S. G. Filler, and B. Hube.** 2008. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog.* **4:**e1000217.
2. **Argimon, S., J. A. Wishart, R. Leng, S. Macaskill, A. Mavor, T. Alexandris, S. Nicholls, A. W. Knight, B. Enjalbert, R. Walmsley, F. C. Odds, N. A. Gow, and A. J. Brown.** 2007. Developmental regulation of an adhesin gene during cellular morphogenesis in the fungal pathogen *Candida albicans*. *Eukaryot. Cell* **6:**682–692.
3. **Austin, S. J., W. Fujimoto, K. W. Marvin, T. M. Vollberg, L. Lorand, and A. M. Jetten.** 1996. Cloning and regulation of cornifin β, a new member of the cornifin/spr family. Suppression by retinoic acid receptor-selective retinoids. *J. Biol. Chem.* **271:**3737–3742.
4. **Bastidas, R. J., J. Heitman, and M. E. Cardenas.** 2009. The protein kinase Tor1 regulates adhesin gene expression in *Candida albicans*. *PLoS Pathog.* **5:**e1000294.
5. **Bester, M. C., I. S. Pretorius, and F. F. Bauer.** 2006. The regulation of *Saccharomyces cerevisiae* FLO gene expression and Ca2+-dependent flocculation by Flo8p and Mss11p. *Curr. Genet.* **49:**375–383.
6. **Bockmuhl, D. P., and J. F. Ernst.** 2001. A potential phosphorylation site for an A-type kinase in the Efg1 regulator protein contributes to hyphal morphogenesis of *Candida albicans*. *Genetics* **157:**1523–1530.
7. **Braun, B. R., and A. D. Johnson.** 2000. TUP1, CPH1 and EFG1 make independent contributions to filamentation in *Candida albicans*. *Genetics* **155:**57–67.
8. **Braus, G. H., O. Grundmann, S. Bruckner, and H. U. Mosch.** 2003. Amino acid starvation and Gcn4p regulate adhesive growth and FLO11 gene expression in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **14:**4272–4284.
9. **Brown, C. A., A. W. Murray, and K. J. Verstrepen.** 2010. Rapid expansion and functional divergence of subtelomeric gene families in yeasts. *Curr. Biol.* **20:**895–903.
10. **Bumgarner, S. L., R. D. Dowell, P. Grisafi, D. K. Gifford, and G. R. Fink.** 2009. Toggle involving cis-interfering noncoding RNAs controls variegated gene expression in yeast. *Proc. Natl. Acad. Sci. USA* **106:**18321–18326.
11. **Castano, I., S. J. Pan, M. Zupancic, C. Hennequin, B. Dujon, and B. P. Cormack.** 2005. Telomere length control and transcriptional regulation of subtelomeric adhesins in *Candida glabrata*. *Mol. Microbiol.* **55:**1246–1258.
12. **Chandra, J., D. M. Kuhn, P. K. Mukherjee, L. L. Hoyer, T. McCormick, and M. A. Ghannoum.** 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J. Bacteriol.* **183:**5385–5394.
13. **Cheng, G., K. Wozniak, M. A. Wallig, P. L. Fidel, Jr., S. R. Trupin, and L. L. Hoyer.** 2005. Comparison between *Candida albicans* agglutinin-like sequence gene expression patterns in human clinical specimens and models of vaginal candidiasis. *Infect. Immun.* **73:**1656–1663.
14. **Coleman, D. A., S. H. Oh, X. Zhao, H. Zhao, J. T. Hutchins, J. H. Vernachio, J. M. Patti, and L. L. Hoyer.** 2009. Monoclonal antibodies specific for *Candida albicans* Als3 that immunolabel fungal cells in vitro and in vivo and block adhesion to host surfaces. *J. Microbiol. Methods* **78:**71–78.
15. **Cormack, B. P., N. Ghori, and S. Falkow.** 1999. An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285:**578–582.
16. **Cullen, P. J., and G. F. Sprague, Jr.** 2000. Glucose depletion causes haploid invasive growth in yeast. *Proc. Natl. Acad. Sci. USA* **97:**13619–13624.
17. **Daniels, K. J., S. R. Lockhart, J. F. Staab, P. Sundstrom, and D. R. Soll.** 2003. The adhesin Hwp1 and the first daughter cell localize to the a/a portion of the conjugation bridge during *Candida albicans* mating. *Mol. Biol. Cell* **14:**4920–4930.

18. **de Boer, A. D., P. W. de Groot, G. Weindl, M. Schaller, D. Riedel, R. Diez-Orejas, F. M. Klis, C. G. de Koster, H. L. Dekker, U. Gross, O. Bader, and M. Weig.** 2010. The *Candida albicans* cell wall protein Rhd3/Pga29 is abundant in the yeast form and contributes to virulence. *Yeast* **27:**611–624.
19. **de Groot, P. W., A. D. de Boer, J. Cunningham, H. L. Dekker, L. de Jong, K. J. Hellingwerf, C. de Koster, and F. M. Klis.** 2004. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryot. Cell* **3:**955–965.
20. **de Groot, P. W., K. J. Hellingwerf, and F. M. Klis.** 2003. Genome-wide identification of fungal GPI proteins. *Yeast* **20:**781–796.
21. **de Groot, P. W., E. A. Kraneveld, Q. Y. Yin, H. L. Dekker, U. Gross, W. Crielaard, C. G. de Koster, O. Bader, F. M. Klis, and M. Weig.** 2008. The cell wall of the human pathogen *Candida glabrata*: differential incorporation of novel adhesin-like wall proteins. *Eukaryot. Cell* **7:**1951–1964.
22. **De Las Penas, A., S. J. Pan, I. Castano, J. Alder, R. Cregg, and B. P. Cormack.** 2003. Virulence-related surface glycoproteins in the yeast pathogen *Candida glabrata* are encoded in subtelomeric clusters and subject to RAP1- and SIR-dependent transcriptional silencing. *Genes Dev.* **17:**2245–2258.
23. **de Nobel, J. G., F. M. Klis, J. Priem, T. Munnik, and H. van den Ende.** 1990. The glucanase-soluble mannoproteins limit cell wall porosity in *Saccharomyces cerevisiae*. *Yeast* **6:**491–499.
24. **Domergue, R., I. Castano, A. De Las Penas, M. Zupancic, V. Lockatell, J. R. Hebel, D. Johnson, and B. P. Cormack.** 2005. Nicotinic acid limitation regulates silencing of *Candida* adhesins during UTI. *Science* **308:**866–870.
25. **Ene, I. V., and R. J. Bennett.** 2009. Hwp1 and related adhesins contribute to both mating and biofilm formation in *Candida albicans*. *Eukaryot. Cell* **8:**1909–1913.
26. **Fankhauser, N., and P. Maser.** 2005. Identification of GPI anchor attachment signals by a Kohonen self-organizing map. *Bioinformatics* **21:**1846–1852.
27. **Fichtner, L., F. Schulze, and G. H. Braus.** 2007. Differential Flo8p-dependent regulation of FLO1 and FLO11 for cell-cell and cell-substrate adherence of *S. cerevisiae* S288c. *Mol. Microbiol.* **66:**1276–1289.
28. **Fidalgo, M., R. R. Barrales, J. I. Ibeas, and J. Jimenez.** 2006. Adaptive evolution by mutations in the FLO11 gene. *Proc. Natl. Acad. Sci. USA* **103:**11228–11233.
29. **Filler, S. G., J. N. Swerdloff, C. Hobbs, and P. M. Luckett.** 1995. Penetration and damage of endothelial cells by *Candida albicans*. *Infect. Immun.* **63:**976–983.
30. **Frank, A. T., C. B. Ramsook, H. N. Otoo, C. Tan, G. Soybelman, J. M. Rauceo, N. K. Gaur, S. A. Klotz, and P. N. Lipke.** 2010. Structure and function of glycosylated tandem repeats from *Candida albicans* Als adhesins. *Eukaryot. Cell* **9:**405–414.
31. **Frieman, M. B., J. M. McCaffery, and B. P. Cormack.** 2002. Modular domain structure in the *Candida glabrata* adhesin Epa1p, a β1,6 glucan-cross-linked cell wall protein. *Mol. Microbiol.* **46:**479–492.
32. **Fu, Y., A. S. Ibrahim, D. C. Sheppard, Y. C. Chen, S. W. French, J. E. Cutler, S. G. Filler, and J. E. Edwards, Jr.** 2002. *Candida albicans* Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. *Mol. Microbiol.* **44:**61–72.
33. **Gagiano, M., M. Bester, D. van Dyk, J. Franken, F. F. Bauer, and I. S. Pretorius.** 2003. Mss11p is a transcription factor regulating pseudohyphal differentiation, invasive growth and starch metabolism in *Saccharomyces cerevisiae* in response to nutrient availability. *Mol. Microbiol.* **47:**119–134.
34. **Garcia-Sanchez, S., S. Aubert, I. Iraqui, G. Janbon, J. M. Ghigo, and C. d'Enfert.** 2004. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot. Cell* **3:**536–545.
35. **Gaur, N. K., S. A. Klotz, and R. L. Henderson.** 1999. Overexpression of the *Candida albicans ALA1* gene in *Saccharomyces cerevisiae* results in aggregation following attachment of yeast cells to extracellular matrix proteins, adherence properties similar to those of *Candida albicans*. *Infect. Immun.* **67:**6040–6047.
36. **Govender, P., J. L. Domingo, M. C. Bester, I. S. Pretorius, and F. F. Bauer.** 2008. Controlled expression of the dominant flocculation genes *FLO1*, *FLO5*, and *FLO11* in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **74:**6041–6052.
37. **Green, C. B., G. Cheng, J. Chandra, P. Mukherjee, M. A. Ghannoum, and L. L. Hoyer.** 2004. RT-PCR detection of *Candida albicans* ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. *Microbiology* **150:**267–275.
38. **Green, C. B., S. M. Marretta, G. Cheng, F. F. Faddoul, E. J. Ehrhart, and L. L. Hoyer.** 2006. RT-PCR analysis of *Candida albicans* ALS gene expression in a hyposalivatory rat model of oral candidiasis and in HIV-positive human patients. *Med. Mycol.* **44:**103–111.
39. **Green, C. B., X. Zhao, K. M. Yeater, and L. L. Hoyer.** 2005. Construction and real-time RT-PCR validation of *Candida albicans* PALS-GFP reporter strains and their use in flow cytometry analysis of ALS gene expression in budding and filamenting cells. *Microbiology* **151:**1051–1060.
40. **Green, S. R., and A. D. Johnson.** 2004. Promoter-dependent roles for the Srb10 cyclin-dependent kinase and the Hda1 deacetylase in Tup1-mediated repression in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **15:**4191–4202.
41. **Guo, B., C. A. Styles, Q. Feng, and G. R. Fink.** 2000. A *Saccharomyces* gene family involved in invasive growth, cell-cell adhesion, and mating. *Proc. Natl. Acad. Sci. USA* **97:**12158–12163.
42. **Halme, A., S. Bumgarner, C. Styles, and G. R. Fink.** 2004. Genetic and epigenetic regulation of the FLO gene family generates cell-surface variation in yeast. *Cell* **116:**405–415.
43. **Heringa, J., and P. Argos.** 1993. A method to recognize distant repeats in protein sequences. *Proteins* **17:**391–441.
44. **Hoyer, L. L.** 2001. The ALS gene family of *Candida albicans*. *Trends Microbiol.* **9:**176–180.
45. **Hoyer, L. L., C. B. Green, S. H. Oh, and X. Zhao.** 2008. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family—a sticky pursuit. *Med. Mycol.* **46:**1–15.
46. **Hoyer, L. L., and J. E. Hecht.** 2001. The ALS5 gene of *Candida albicans* and analysis of the Als5p N-terminal domain. *Yeast* **18:**49–60.
47. **Hoyer, L. L., and J. E. Hecht.** 2000. The ALS6 and ALS7 genes of *Candida albicans*. *Yeast* **16:**847–855.
48. **Hoyer, L. L., T. L. Payne, M. Bell, A. M. Myers, and S. Scherer.** 1998. *Candida albicans* ALS3 and insights into the nature of the ALS gene family. *Curr. Genet.* **33:**451–459.
49. **Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi.** 1995. *Candida albicans* ALS1: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. *Mol. Microbiol.* **15:**39–54.
50. **Ibrahim, A. S., B. J. Spellberg, V. Avanesian, Y. Fu, and J. E. Edwards, Jr.** 2006. The anti-*Candida* vaccine based on the recombinant N-terminal domain of Als1p is broadly active against disseminated candidiasis. *Infect. Immun.* **74:**3039–3041.

51. **Ibrahim, A. S., B. J. Spellberg, V. Avenissian, Y. Fu, S. G. Filler, and J. E. Edwards, Jr.** 2005. Vaccination with recombinant N-terminal domain of Als1p improves survival during murine disseminated candidiasis by enhancing cell-mediated, not humoral, immunity. *Infect. Immun.* **73:** 999–1005.
52. **Iraqui, I., S. Garcia-Sanchez, S. Aubert, F. Dromer, J. M. Ghigo, C. d'Enfert, and G. Janbon.** 2005. The Yak1p kinase controls expression of adhesins and biofilm formation in *Candida glabrata* in a Sir4p-dependent pathway. *Mol. Microbiol.* **55:**1259–1271.
53. **Kadosh, D., and A. D. Johnson.** 2005. Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: a genome-wide analysis. *Mol. Biol. Cell* **16:**2903–2912.
54. **Kang, S., and H. Choi.** 2005. Effect of surface hydrophobicity on the adhesion of *S. cerevisiae* onto modified surfaces by poly(styrene-ran-sulfonic acid) random copolymers. *Colloids Surf. B Biointerfaces* **46:**70–77.
55. **Kapteyn, J. C., L. L. Hoyer, J. E. Hecht, W. H. Muller, A. Andel, A. J. Verkleij, M. Makarow, H. Van Den Ende, and F. M. Klis.** 2000. The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. *Mol. Microbiol.* **35:**601–611.
56. **Kapteyn, J. C., H. Van Den Ende, and F. M. Klis.** 1999. The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochim. Biophys. Acta* **1426:**373–383.
57. **Kaur, R., R. Domergue, M. L. Zupancic, and B. P. Cormack.** 2005. A yeast by any other name: *Candida glabrata* and its interaction with the host. *Curr. Opin. Microbiol.* **8:**378–384.
58. **Klis, F. M.** 1994. Review: cell wall assembly in yeast. *Yeast* **10:**851–869.
59. **Klis, F. M., A. Boorsma, and P. W. De Groot.** 2006. Cell wall construction in *Saccharomyces cerevisiae*. *Yeast* **23:** 185–202.
60. **Klis, F. M., L. H. Caro, J. H. Vossen, J. C. Kapteyn, A. F. Ram, R. C. Montijn, M. A. Van Berkel, and H. Van den Ende.** 1997. Identification and characterization of a major building block in the cell wall of *Saccharomyces cerevisiae*. *Biochem. Soc. Trans.* **25:**856–860.
61. **Klotz, S. A., N. K. Gaur, D. F. Lake, V. Chan, J. Rauceo, and P. N. Lipke.** 2004. Degenerate peptide recognition by *Candida albicans* adhesins Als5p and Als1p. *Infect. Immun.* **72:**2029–2034.
62. **Kobayashi, O., N. Hayashi, R. Kuroki, and H. Sone.** 1998. Region of FLO1 proteins responsible for sugar recognition. *J. Bacteriol.* **180:**6503–6510.
63. **Kobayashi, O., H. Yoshimoto, and H. Sone.** 1999. Analysis of the genes activated by the FLO8 gene in *Saccharomyces cerevisiae*. *Curr. Genet.* **36:**256–261.
64. **Kollar, R., B. B. Reinhold, E. Petrakova, H. J. Yeh, G. Ashwell, J. Drgonova, J. C. Kapteyn, F. M. Klis, and E. Cabib.** 1997. Architecture of the yeast cell wall: β(1→6)-glucan interconnects mannoprotein, β(1→3)-glucan, and chitin. *J. Biol. Chem.* **272:**17762–17775.
65. **Kumamoto, C. A., and M. D. Vinces.** 2005. Contributions of hyphae and hypha-co-regulated genes to *Candida albicans* virulence. *Cell. Microbiol.* **7:**1546–1554.
66. **Leng, P., P. R. Lee, H. Wu, and A. J. Brown.** 2001. Efg1, a morphogenetic regulator in *Candida albicans*, is a sequence-specific DNA binding protein. *J. Bacteriol.* **183:** 4090–4093.
67. **Li, F., and S. P. Palecek.** 2008. Distinct domains of the *Candida albicans* adhesin Eap1p mediate cell-cell and cell-substrate interactions. *Microbiology* **154:**1193–1203.
68. **Li, F., and S. P. Palecek.** 2003. *EAP1*, a *Candida albicans* gene involved in binding human epithelial cells. *Eukaryot. Cell* **2:**1266–1273.
69. **Li, F., and S. P. Palecek.** 2005. Identification of *Candida albicans* genes that induce *Saccharomyces cerevisiae* cell adhesion and morphogenesis. *Biotechnol. Prog.***21:**1601–1609.
70. **Li, F., M. J. Svarovsky, A. J. Karlsson, J. P. Wagner, K. Marchillo, P. Oshel, D. Andes, and S. P. Palecek.** 2007. Eap1p, an adhesin that mediates *Candida albicans* biofilm formation in vitro and in vivo. *Eukaryot. Cell* **6:**931–939.
71. **Lipke, P. N., and R. Ovalle.** 1998. Cell wall architecture in yeast: new structure and new challenges. *J. Bacteriol.* **180:**3735–3740.
72. **Liu, H., C. A. Styles, and G. R. Fink.** 1996. *Saccharomyces cerevisiae* S288C has a mutation in FLO8, a gene required for filamentous growth. *Genetics* **144:**967–978.
73. **Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90:**939–949.
74. **Lott, T. J., B. P. Holloway, D. A. Logan, R. Fundyga, and J. Arnold.** 1999. Towards understanding the evolution of the human commensal yeast *Candida albicans*. *Microbiology* **145**(Pt. 5)**:**1137–1143.
75. **Loza, L., Y. Fu, A. S. Ibrahim, D. C. Sheppard, S. G. Filler, and J. E. Edwards, Jr.** 2004. Functional analysis of the *Candida albicans* ALS1 gene product. *Yeast* **21:**473–482.
76. **Ma, B., S. J. Pan, R. Domergue, T. Rigby, M. Whiteway, D. Johnson, and B. P. Cormack.** 2009. High-affinity transporters for NAD^+ precursors in *Candida glabrata* are regulated by Hst1 and induced in response to niacin limitation. *Mol. Cell. Biol.* **29:**4067–4079.
77. **Ma, B., S. J. Pan, M. L. Zupancic, and B. P. Cormack.** 2007. Assimilation of NAD^+ precursors in *Candida glabrata*. *Mol. Microbiol.* **66:**14–25.
78. **MacCallum, D. M., L. Castillo, K. Nather, C. A. Munro, A. J. Brown, N. A. Gow, and F. C. Odds.** 2009. Property differences among the four major *Candida albicans* strain clades. *Eukaryot. Cell* **8:**373–387.
79. **Moreno-Ruiz, E., M. Galan-Diez, W. Zhu, E. Fernandez-Ruiz, C. d'Enfert, S. G. Filler, P. Cossart, and E. Veiga.** 2009. *Candida albicans* internalization by host cells is mediated by a clathrin-dependent mechanism. *Cell. Microbiol.* **11:**1179–1189.
80. **Mundy, R. D., and B. Cormack.** 2009. Expression of *Candida glabrata* adhesins after exposure to chemical preservatives. *J. Infect. Dis.* **199:**1891–1898.
81. **Naglik, J. R., F. Fostira, J. Ruprai, J. F. Staab, S. J. Challacombe, and P. Sundstrom.** 2006. *Candida albicans* HWP1 gene expression and host antibody responses in colonization and disease. *J. Med. Microbiol.* **55:**1323–1327.
82. **Nailis, H., S. Kucharikova, M. Ricicova, P. Van Dijck, D. Deforce, H. Nelis, and T. Coenye.** 2010. Real-time PCR expression profiling of genes encoding potential virulence factors in *Candida albicans* biofilms: identification of model-dependent and -independent gene expression. *BMC Microbiol.* **10:**114.
83. **Nailis, H., R. Vandenbroucke, K. Tilleman, D. Deforce, H. Nelis, and T. Coenye.** 2009. Monitoring ALS1 and ALS3 gene expression during in vitro *Candida albicans* biofilm formation under continuous flow conditions. *Mycopathologia* **167:**9–17.
84. **Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell.** 2006. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation in vitro and in vivo. *PLoS Pathog.* **2:**e63.
85. **Nobile, C. J., and A. P. Mitchell.** 2005. Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. *Curr. Biol.* **15:**1150–1155.
86. **Nobile, C. J., J. E. Nett, D. R. Andes, and A. P. Mitchell.** 2006. Function of *Candida albicans* adhesin Hwp1 in biofilm formation. *Eukaryot. Cell* **5:**1604–1610.

87. **Nobile, C. J., H. A. Schneider, J. E. Nett, D. C. Sheppard, S. G. Filler, D. R. Andes, and A. P. Mitchell.** 2008. Complementary adhesin function in *C. albicans* biofilm formation. *Curr. Biol.* **18:**1017–1024.
88. **O'Connor, L., S. Lahiff, F. Casey, M. Glennon, M. Cormican, and M. Maher.** 2005. Quantification of ALS1 gene expression in *Candida albicans* biofilms by RT-PCR using hybridisation probes on the LightCycler. *Mol. Cell. Probes* **19:**153–162.
89. **Oh, S. H., G. Cheng, J. A. Nuessen, R. Jajko, K. M. Yeater, X. Zhao, C. Pujol, D. R. Soll, and L. L. Hoyer.** 2005. Functional specificity of *Candida albicans* Als3p proteins and clade specificity of ALS3 alleles discriminated by the number of copies of the tandem repeat sequence in the central domain. *Microbiology* **151:**673–681.
90. **Otoo, H. N., K. G. Lee, W. Qiu, and P. N. Lipke.** 2008. *Candida albicans* Als adhesins have conserved amyloid-forming sequences. *Eukaryot. Cell* **7:**776–782.
91. **Pan, X., and J. Heitman.** 2002. Protein kinase A operates a molecular switch that governs yeast pseudohyphal differentiation. *Mol. Cell. Biol.* **22:**3981–3993.
92. **Park, H., C. L. Myers, D. C. Sheppard, Q. T. Phan, A. A. Sanchez, J. E. Edwards, and S. G. Filler.** 2005. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. *Cell. Microbiol.* **7:**499–510.
93. **Phan, Q. T., P. H. Belanger, and S. G. Filler.** 2000. Role of hyphal formation in interactions of *Candida albicans* with endothelial cells. *Infect. Immun.* **68:** 3485–3490.
94. **Phan, Q. T., C. L. Myers, Y. Fu, D. C. Sheppard, M. R. Yeaman, W. H. Welch, A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol.* **5:**e64.
95. **Rachidi, N., M. J. Martinez, P. Barre, and B. Blondin.** 2000. *Saccharomyces cerevisiae* PAU genes are induced by anaerobiosis. *Mol. Microbiol.* **35:**1421–1430.
96. **Ramsook, C. B., C. Tan, M. C. Garcia, R. Fung, G. Soybelman, R. Henry, A. Litewka, S. O'Meally, H. N. Otoo, R. A. Khalaf, A. M. Dranginis, N. K. Gaur, S. A. Klotz, J. M. Rauceo, C. K. Jue, and P. N. Lipke.** 2010. Yeast cell adhesion molecules have functional amyloid-forming sequences. *Eukaryot. Cell* **9:**393–404.
97. **Rauceo, J. M., R. De Armond, H. Otoo, P. C. Kahn, S. A. Klotz, N. K. Gaur, and P. N. Lipke.** 2006. Threonine-rich repeats increase fibronectin binding in the *Candida albicans* adhesin Als5p. *Eukaryot. Cell* **5:** 1664–1673.
98. **Rigden, D. J., L. V. Mello, and M. Y. Galperin.** 2004. The PA14 domain, a conserved all-beta domain in bacterial toxins, enzymes, adhesins and signaling molecules. *Trends Biochem. Sci.* **29:**335–339.
99. **Roberts, R. L., and G. R. Fink.** 1994. Elements of a single MAP kinase cascade in *Saccharomyces cerevisiae* mediate two developmental programs in the same cell type: mating and invasive growth. *Genes Dev.* **8:**2974–2985.
100. **Rolland, T., B. Dujon, and G. F. Richard.** 2010. Dynamic evolution of megasatellites in yeasts. *Nucleic Acids Res.* **38:**4731–4739.
101. **Rosas-Hernandez, L. L., A. Juarez-Reyes, O. E. Arroyo-Helguera, A. De Las Penas, S. J. Pan, B. P. Cormack, and I. Castano.** 2008. yKu70/yKu80 and Rif1 regulate silencing differentially at telomeres in *Candida glabrata*. *Eukaryot. Cell* **7:**2168–2178.
102. **Rupp, S., E. Summers, H. J. Lo, H. Madhani, and G. Fink.** 1999. MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast FLO11 gene. *EMBO J.* **18:**1257–1269.
103. **Sanchez, A. A., D. A. Johnston, C. Myers, J. E. Edwards, Jr., A. P. Mitchell, and S. G. Filler.** 2004. Relationship between *Candida albicans* virulence during experimental hematogenously disseminated infection and endothelial cell damage in vitro. *Infect. Immun.* **72:** 598–601.
104. **Sato, M., H. Maeba, J. Watari, and M. Takashio.** 2002. Analysis of an inactivated Lg-FLO1 gene present in bottom-fermenting yeast. *J. Biosci. Bioeng.* **93:**395–398.
105. **Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu, A. S. Ibrahim, S. G. Filler, M. Zhang, A. J. Waring, and J. E. Edwards, Jr.** 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. *J. Biol. Chem.* **279:**30480–30489.
106. **Sherman, D. J., T. Martin, M. Nikolski, C. Cayla, J. L. Souciet, and P. Durrens.** 2009. Genolevures: protein families and synteny among complete hemiascomycetous yeast proteomes and genomes. *Nucleic Acids Res.* **37:** D550–D554.
107. **Skrzypek, M. S., M. B. Arnaud, M. C. Costanzo, D. O. Inglis, P. Shah, G. Binkley, S. R. Miyasato, and G. Sherlock.** 2010. New tools at the *Candida* Genome Database: biochemical pathways and full-text literature search. *Nucleic Acids Res.* **38:**D428–D432.
108. **Smith, R. L., and A. D. Johnson.** 2000. Turning genes off by Ssn6-Tup1: a conserved system of transcriptional repression in eukaryotes. *Trends Biochem. Sci.* **25:** 325–330.
109. **Sonneborn, A., D. P. Bockmuhl, M. Gerads, K. Kurpanek, D. Sanglard, and J. F. Ernst.** 2000. Protein kinase A encoded by TPK2 regulates dimorphism of *Candida albicans*. *Mol. Microbiol.* **35:**386–396.
110. **Spellberg, B., A. S. Ibrahim, M. R. Yeaman, L. Lin, Y. Fu, V. Avanesian, A. S. Bayer, S. G. Filler, P. Lipke, H. Otoo, and J. E. Edwards, Jr.** 2008. The antifungal vaccine derived from the recombinant N terminus of Als3p protects mice against the bacterium *Staphylococcus aureus*. *Infect. Immun.* **76:**4574–4580.
111. **Spellberg, B. J., A. S. Ibrahim, V. Avanesian, Y. Fu, C. Myers, Q. T. Phan, S. G. Filler, M. R. Yeaman, and J. E. Edwards, Jr.** 2006. Efficacy of the anti-*Candida* rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J. Infect. Dis.* **194:**256–260.
112. **Spellberg, B. J., A. S. Ibrahim, V. Avenissian, S. G. Filler, C. L. Myers, Y. Fu, and J. E. Edwards, Jr.** 2005. The anti-*Candida albicans* vaccine composed of the recombinant N terminus of Als1p reduces fungal burden and improves survival in both immunocompetent and immunocompromised mice. *Infect. Immun.* **73:**6191–6193.
113. **Staab, J. F., Y. S. Bahn, C. H. Tai, P. F. Cook, and P. Sundstrom.** 2004. Expression of transglutaminase substrate activity on *Candida albicans* germ tubes through a coiled, disulfide-bonded N-terminal domain of Hwp1 requires C-terminal glycosylphosphatidylinositol modification. *J. Biol. Chem.* **279:**40737–40747.
114. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283:**1535–1538.
115. **Staab, J. F., C. A. Ferrer, and P. Sundstrom.** 1996. Developmental expression of a tandemly repeated, proline- and glutamine-rich amino acid motif on hyphal surfaces on *Candida albicans*. *J. Biol. Chem.* **271:**6298–6305.
116. **Staab, J. F., and P. Sundstrom.** 1998. Genetic organization and sequence analysis of the hypha-specific cell wall protein gene HWP1 of *Candida albicans*. *Yeast* **14:** 681–686.
117. **Stoldt, V. R., A. Sonneborn, C. E. Leuker, and J. F. Ernst.** 1997. Efg1p, an essential regulator of morphogen-

esis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J.* **16:**1982–1991.
118. **Sundstrom, P.** 2002. Adhesion in *Candida* spp. *Cell. Microbiol.* **4:**461–469.
119. **Sundstrom, P., E. Balish, and C. M. Allen.** 2002. Essential role of the *Candida albicans* transglutaminase substrate, hyphal wall protein 1, in lethal oroesophageal candidiasis in immunodeficient mice. *J. Infect. Dis.* **185:** 521–530.
120. **Sundstrom, P., J. E. Cutler, and J. F. Staab.** 2002. Reevaluation of the role of *HWP1* in systemic candidiasis by use of *Candida albicans* strains with selectable marker *URA3* targeted to the *ENO1* locus. *Infect. Immun.* **70:** 3281–3283.
121. **Thierry, A., C. Bouchier, B. Dujon, and G. F. Richard.** 2008. Megasatellites: a peculiar class of giant minisatellites in genes involved in cell adhesion and pathogenicity in *Candida glabrata*. *Nucleic Acids Res.* **36:**5970–5982.
122. **Thierry, A., B. Dujon, and G. F. Richard.** 2010. Megasatellites: a new class of large tandem repeats discovered in the pathogenic yeast *Candida glabrata*. *Cell. Mol. Life Sci.* **67:**671–676.
123. **Van Mulders, S. E., E. Christianen, S. M. Saerens, L. Daenen, P. J. Verbelen, R. Willaert, K. J. Verstrepen, and F. R. Delvaux.** 2009. Phenotypic diversity of Flo protein family-mediated adhesion in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **9:**178–190.
124. **Verstrepen, K. J., A. Jansen, F. Lewitter, and G. R. Fink.** 2005. Intragenic tandem repeats generate functional variability. *Nat. Genet.* **37:**986–990.
125. **Verstrepen, K. J., and F. M. Klis.** 2006. Flocculation, adhesion and biofilm formation in yeasts. *Mol. Microbiol.* **60:**5–15.
126. **Verstrepen, K. J., T. B. Reynolds, and G. R. Fink.** 2004. Origins of variation in the fungal cell surface. *Nat. Rev. Microbiol.* **2:**533–540.
127. **Wapinski, I., A. Pfeffer, N. Friedman, and A. Regev.** 2007. Natural history and evolutionary principles of gene duplication in fungi. *Nature* **449:**54–61.
128. **Weig, M., L. Jansch, U. Gross, C. G. De Koster, F. M. Klis, and P. W. De Groot.** 2004. Systematic identification in silico of covalently bound cell wall proteins and analysis of protein-polysaccharide linkages of the human pathogen *Candida glabrata*. *Microbiology* **150:** 3129–3144.
129. **Wheeler, R. T., D. Kombe, S. D. Agarwala, and G. R. Fink.** 2008. Dynamic, morphotype-specific *Candida albicans* beta-glucan exposure during infection and drug treatment. *PLoS Pathog.* **4:**e1000227.
130. **Wheeler, R. T., M. Kupiec, P. Magnelli, C. Abeijon, and G. R. Fink.** 2003. A *Saccharomyces cerevisiae* mutant with increased virulence. *Proc. Natl. Acad. Sci. USA* **100:**2766–2770.
131. **Zakikhany, K., J. R. Naglik, A. Schmidt-Westhausen, G. Holland, M. Schaller, and B. Hube.** 2007. In vivo transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. *Cell. Microbiol.* **9:**2938–2954.
132. **Zhang, N., A. L. Harrex, B. R. Holland, L. E. Fenton, R. D. Cannon, and J. Schmid.** 2003. Sixty alleles of the ALS7 open reading frame in *Candida albicans*: ALS7 is a hypermutable contingency locus. *Genome Res.* **13:** 2005–2017.
133. **Zhang, Z., and J. C. Reese.** 2004. Redundant mechanisms are used by Ssn6-Tup1 in repressing chromosomal gene transcription in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279:**39240–39250.
134. **Zhao, X., S. H. Oh, G. Cheng, C. B. Green, J. A. Nuessen, K. Yeater, R. P. Leng, A. J. Brown, and L. L. Hoyer.** 2004. ALS3 and ALS8 represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. *Microbiology* **150:** 2415–2428.
135. **Zhao, X., S. H. Oh, and L. L. Hoyer.** 2007. Deletion of ALS5, ALS6 or ALS7 increases adhesion of *Candida albicans* to human vascular endothelial and buccal epithelial cells. *Med. Mycol.* **45:**429–434.
136. **Zhao, X., S. H. Oh, and L. L. Hoyer.** 2007. Unequal contribution of ALS9 alleles to adhesion between *Candida albicans* and human vascular endothelial cells. *Microbiology* **153:**2342–2350.
137. **Zhao, X., S. H. Oh, R. Jajko, D. J. Diekema, M. A. Pfaller, C. Pujol, D. R. Soll, and L. L. Hoyer.** 2007. Analysis of ALS5 and ALS6 allelic variability in a geographically diverse collection of *Candida albicans* isolates. *Fungal Genet. Biol.* **44:**1298–1309.
138. **Zhao, X., S. H. Oh, K. M. Yeater, and L. L. Hoyer.** 2005. Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* **151:** 1619–1630.
139. **Zhao, X., C. Pujol, D. R. Soll, and L. L. Hoyer.** 2003. Allelic variation in the contiguous loci encoding *Candida albicans* ALS5, ALS1 and ALS9. *Microbiology* **149:** 2947–2960.
140. **Zupancic, M. L., M. Frieman, D. Smith, R. A. Alvarez, R. D. Cummings, and B. P. Cormack.** 2008. Glycan microarray analysis of *Candida glabrata* adhesin ligand specificity. *Mol. Microbiol.* **68:**547–559.

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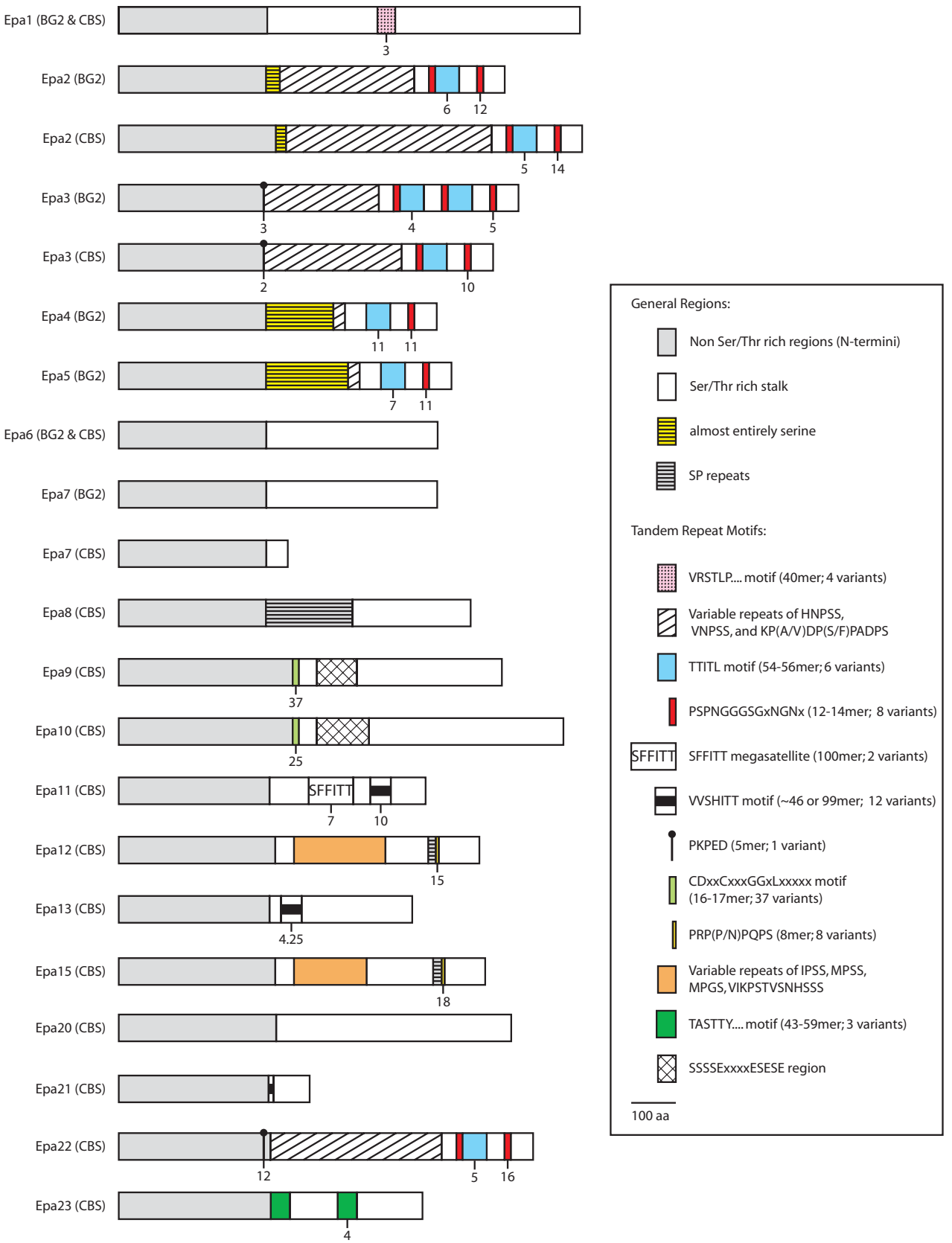
Epa1 (BG2 & CBS)
3
Epa2 (BG2)
6
12
Epa2 (CBS)
5
14
Epa3 (BG2)
3
4
5
Epa3 (CBS)
2
10
Epa4 (BG2)
11
11
Epa5 (BG2)
7
11
Epa6 (BG2 & CBS)
Epa7 (BG2)
Epa7 (CBS)
Epa8 (CBS)
Epa9 (CBS)
37
Epa10 (CBS)
25
Epa11 (CBS)
SFFITT
7
10
Epa12 (CBS)
15
Epa13 (CBS)
4.25
Epa15 (CBS)
18
Epa20 (CBS)
Epa21 (CBS)
Epa22 (CBS)
12
5
16
Epa23 (CBS)
4
General Regions:
Non Ser/Thr rich regions (N-termini)
Ser/Thr rich stalk
almost entirely serine
SP repeats
Tandem Repeat Motifs:
VRSTLP.... motif (40mer; 4 variants)
Variable repeats of HNPSS, VNPSS, and KP(A/V)DP(S/F)PADPS
TTITL motif (54-56mer; 6 variants)
PSPNGGGSGxNGNx (12-14mer; 8 variants)
SFFITT
SFFITT megasatellite (100mer; 2 variants)
VVSHITT motif (~46 or 99mer; 12 variants)
PKPED (5mer; 1 variant)
CDxxCxxxGGxLxxxxx motif (16-17mer; 37 variants)
PRP(P/N)PQPS (8mer; 8 variants)
Variable repeats of IPSS, MPSS, MPGS, VIKPSTVSNHSSS
TASTTY.... motif (43-59mer; 3 variants)
SSSSExxxxESESE region
100 aa

COLOR PLATE 4 (CHAPTER 16) Schematic of protein domains in a subset of the *C. glabrata* Epa adhesins. The section "Structure/function analysis of Epa adhesins" discusses the modular structure of the Epa adhesins. Each drawing is to scale, with TRs collapsed such that one motif unit is shown and the number of repeat units is indicated below the drawing. The key contains the length of each repeat motif and the number of variants observed among all Epa proteins. For shorter motifs, the key also contains the consensus sequence (75% identity among variants). The VRSTLP motif has the consensus VRSTLPSSAGSNETSINVPFSSSTESNTSTSSTSTSNSKx (22). The TASTTY motif has the consensus xTASTTYxPGxVxxxTTISTYxTxITGxDNRxTPETVFVVETP. Certain regions (diagonal lines; orange boxes) contain a mixture of shorter motif units linked together in various combinations; these areas are drawn to indicate the full length of the region and have not been collapsed to represent the shorter sequences. The lattice boxes denote a region rich in Ser and Glu residues, which occasionally has the sequence SSSSExxxxESESE repeated at nonregular intervals. Motifs in Epa1- to -5 have been published previously (22). The VSHITT, SFFIT, and TTITL motifs also have been described previously (21, 121). 10.1128/9781555817176.ch16cp4

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch17

17

Encounters with Mammalian Cells: Survival Strategies of *Candida* Species

SLAVENA VYLKOVA AND MICHAEL C. LORENZ

Candida species have a long evolutionary history with mammals, to the point that several species, including *Candida albicans* and the distantly related *C. glabrata*, are not thought to have natural niches outside the host (50). As a result, they have developed intricate survival mechanisms that account for their unique success as eukaryotic organisms that exist as both commensals and pathogens. The concept of *Candida* evolving in the context of the mammalian host is really a story of the fungus developing specific interactions and responses with different mammalian cell types, especially those of the innate immune system. This system, which includes both the passive barriers of epithelial and endothelial layers and the phagocytic cells that confront invasive cells, represents the bulk of our antifungal defenses.

The mammalian immune system is a formidable barrier to the propagation of microbes within the body. As opportunistic pathogens, *Candida* species generally cannot overcome the defenses posed by a healthy immune system (with a few exceptions); rather, expansion of the *Candida* population and resulting pathology require some impairment of immune function. Importantly, even a modest debilitation can increase the risk for disseminated fungal infections: a weakened immune system is not the same as a nonexistent one, so the pathology of candidiasis and the prospects for improved therapeutics are directly related to the interaction of *Candida* with host cells. This chapter reviews the changes in cellular physiology that follow contact with host cells and the impact of these changes on the host-pathogen interaction. The focus is on *C. albicans*, for the simple reason that the vast majority of the published research is on this species, but relevant findings from other species are included where available.

RELEVANT CELL TYPES

This chapter focuses on the interaction between *Candida* cells and the most important and relevant mammalian cell types, introduced briefly below. In its commensal state in nonsterile sites in the mammalian body, *C. albicans* is in constant contact with the epithelial cells that line mucosal surfaces and also various components of the immune system originating from gut-associated lymphoid tissues. As it invades beyond the epithelium, the fungal cell will encounter a greater number and variety of immune cells, particularly the phagocytes of the innate immune system. Both entry into and exit from the blood, critical steps in dissemination, require *Candida* cells to transit endothelial cell layers. Thus, in a nutshell, the most important host cells in *Candida* pathogenesis are phagocytes and endothelial and epithelial cells, and *C. albicans* has specific responses to each. This is not to say that other cell types are not relevant to pathogenesis (or commensalism), but there are relatively few data regarding these interactions.

Phagocytes

Epidemiological studies of patients with candidiasis make it clear that defects in phagocyte function are a key risk factor, particularly neutropenia (86). The primary focus of *Candida*-phagocyte interactions has been on polymorphonuclear leukocytes (referred to here as neutrophils) and macrophages. Both these cell types phagocytose *Candida* cells in isolated tissue culture models, based on pattern recognition receptors such as dectin-1 (β-glucan), mannose receptor, and Toll-like receptors. Activation of these cells induces a respiratory burst, production of hydrolytic enzymes, and other killing mechanisms (see chapter 11). There are, of course, important differences in potency, life span, tissue tropism, and other functions between neutrophils and macrophages, and as will be seen below, the responses of *C. albicans* reflect this, with a limited overlap between the two. An important technical difference is the availability of macrophage-like cell lines that facilitate in vitro studies, most commonly the mouse-derived RAW264.7 and J774 lines. Though their antifungal activity is limited compared to that of primary cells, their ease of use is an attractive advantage. Similar neutrophil cell lines do not exist, although a neutrophil precursor cell line is available but requires activation for phagocytosis and killing experiments; consequently, these experiments must be done with primary cells,

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usually of human origin. There has been some debate in the literature about the role of dendritic cells, which are primarily antigen-presenting cells. They also phagocytose and kill *Candida*, but at a lower efficiency than macrophages or neutrophils (153). Reflecting the scope of available literature, this chapter concentrates on *Candida* interactions with neutrophils and macrophages.

Endothelial and Epithelial Cells

The broad and generic categories of endothelial and epithelial cells belie a spectrum of subtypes that contact *Candida* cells. Epithelial layers separate nonsterile sites of commensal colonization from sterile sites, and so invasive infections begin with *Candida* cells penetrating this layer. Epithelial surfaces are also the sites for mucosal infections like vaginitis and thrush and other infections of the skin, nails, esophagus, and urinary tract. Endothelial cells are a barrier to dissemination: pathogens must pass in and out of the bloodstream as they spread to tissues. Numerous subtypes of both cells have been used as in vitro models, and notable differences have been observed; for instance, vascular endothelial cells, human umbilical vein cells, and brain microvascular endothelial cells all endocytose *C. albicans*, but brain microvascular endothelial cells appear to be resistant to the tissue damage resulting from hyphal morphogenesis in the other cell types (104, 114, 163). Similarly, a variety of epithelial cell models have been used: corneocytes, buccal cells, HeLa cells, FaDu oral cells, HEp-2 cells, etc. Recently, commercially available human epithelial layers have become more popular, as they are relatively easy to propagate, and adhesion, invasion, and tissue damage appear to correlate with in vivo results (197, 198). To date, there is limited understanding about the relevant differences between these subtypes and how the response of *Candida* might differ between them.

TRANSCRIPTOMIC AND PROTEOMIC ANALYSES

Given the historic genetic limitations of *Candida* species, *C. albicans* in particular, genome-wide technologies presented a powerful approach to understand host-pathogen interactions in detail. As described above, the in vitro coculture systems developed to study the interaction of *C. albicans* with several host cell types are facile, relatively inexpensive, controllable, and scalable, making them easily adaptable to genomic-scale experiments. It is thus not surprising that such experiments were among the first reported as microarrays and proteomics became accessible for *Candida*. The projects described below were not the first attempts to understand the host-pathogen relationship, but they touch on many of the subjects to be discussed in this chapter and are worth describing in some detail at the outset.

The gene expression profiles of *C. albicans* exposed to a variety of mammalian cell types, including whole blood, neutrophils, macrophages, epithelial cells, and endothelial cells, are detailed in Table 1 (70, 71, 130, 163, 188, 190). While there are some overlaps between the phagocyte data sets, it is striking that the responses of *C. albicans* to these different cell types are quite unique. For instance, the sets of differentially regulated genes in response to epithelial versus endothelial cells contain only 29 genes (out of several hundred genes) in common. An analysis of a subset of these genes revealed only one, *VPS51*, involved in vacuolar protein sorting, whose mutation significantly reduced the ability of *C. albicans* to damage both endothelial and epithelial cells (163).

Similarly, the respiratory burst of both neutrophils and (more weakly) macrophages induces in *C. albicans* an oxidative stress response, a major component of the antimicrobial responses of these phagocytes. Beyond this, cells ingested by macrophages display an extensive similarity to cells growing on nonfermentable carbon sources, including the downregulation of protein synthesis and glycolysis and the upregulation of multiple catabolic pathways for less preferred carbon sources (130). This carbon response is greatly muted in cells phagocytosed by neutrophils; instead, these cells appear to sense amino acid starvation, specifically for arginine, leucine, methionine, cysteine, and lysine (70, 188).

Profiling of *C. glabrata* inside macrophages revealed a response broadly similar to that of *C. albicans*, despite the evolutionary distance. Several hundred genes were regulated by phagocytosis, including many involved in carbon metabolism. In addition, some members of an 11-gene family of glycosylphosphatidylinositol (GPI)-anchored "yapsin" proteases were upregulated (see below and reference 106). In two studies the response of *Saccharomyces cerevisiae* was also determined, and in both cases there was some similarity between this model yeast and *C. albicans* or *C. glabrata* (130, 131, 188). However, the response of the pathogenic species was far more robust; 53 genes were differentially regulated in *S. cerevisiae* following macrophage phagocytosis, compared to 545 in *C. albicans* and 616 in *C. glabrata* (106, 130, 131).

One limitation of nucleic acid-based techniques such as DNA microarrays is that the investigator is unable to determine the influence of posttranscriptional modifications or protein degradation rates on the overall phenotype of cells. Fortunately, proteomic analysis of the *Candida*-macrophage interaction is broadly similar to the genomic data, showing an induction of oxidative stress responses and a carbon deprivation profile (65). Phagocytosed cells also induced components of the proteasome, a logical accompaniment to nutrient deprivation but one not detected by DNA microarrays, validating the importance of proteomic analysis.

Efforts have also been made to dissect these complex responses by exposing *C. albicans* to isolated compounds predicted to mediate host-pathogen interactions. This includes components of the antimicrobial burst of phagocytes, such as reactive oxygen species (ROS) (62, 117) and reactive nitrogen species (RNS) (94), and the antimicrobial peptide histatin-5 (223). Other relevant mammalian products have also been tested, including cell-free serum (a potent morphogenetic inducer) (149), prostaglandin E_2 (123), and estrogen derivatives (37).

At the other end of the spectrum, investigators are analyzing gene and protein expression profiles from various in vivo niches, including the kidneys (9, 224), peritoneum (217), biofilms on implanted catheters (154), oral candidiasis from human patients (229), and the gastrointestinal tract (226). These efforts involve a substantial increase in complexity of sample preparation and data analysis and are discussed in more detail in chapter 18.

SPECIFIC RESPONSES TO PHAGOCYTE CONTACT

Morphogenesis

The polymorphic nature of *C. albicans* has attracted great interest throughout the history of studies on this organism,

TABLE 1 Genomic-scale analyses of host-*Candida* interactions[a]

Cell type	Microarray/ proteomics	Major responses[b]	Comments	Reference
With whole cells				
Whole blood	M	Induced: protein synthesis, OSR, glycolysis and glyoxylate cycle, hypha-specific genes	Used unfractionated whole blood with multiple cell types	71
Neutrophils	M	Induced: OSR, amino acid biosynthesis	Isolated human neutrophils	188
Neutrophils	M	Induced: OSR, amino acid biosynthesis Repressed: protein synthesis, glycolysis	Used fractionated human blood; neutrophils represented primary response	70
Macrophages	M	Induced: OSR (mild), glyoxylate cycle/ alternative carbon metabolism Repressed: protein synthesis, glycolysis	Used cultured J774A.1 mouse macrophage line	130
Macrophages	P	Induced: OSR, glyoxylate cycle/ alternative carbon metabolism, proteasome, apoptosis	Used cultured RAW264.7 mouse macrophage line	65
Macrophages (*C. glabrata*)	M	Induced: OSR, glyoxylate cycle/ alternative carbon metabolism, cell wall proteases, Arg/Lys Repressed: glycolysis	Used cultured J774A.1 mouse macrophage line; generally similar to *C. albicans* results	106
Epithelial cells	M	Induced: hyphal specific, *ALS* genes	Used HEp-2 human epithelial cell line	190
Epithelial cells	M	Induced: no statistically significant GO categories Repressed: adhesion (*ALS*), protein synthesis	Used FaDU human oral epithelial cells	163
Endothelial cells	M	No GO categories significantly changed	Used HUVEC	163
Selected in vitro conditions				
Oxidative stress (H_2O_2)	M	Induced: catalase, TRX	Showed lack of a general stress response	62
Oxidative stress (H_2O_2 or diamide)	P	Induced: catalase, TRX, glutathione, other oxidoreductases, some chaperones	Response to diamide and peroxide were similar	117
NO	M	Induced: flavohemoglobin, catalase, glutathione, other oxidoreductases	Some similarities to OSR	94
Histatin-5	M	Induced: stress responses (weakly)	Very limited changes	223
Estrogen (17-β-estradiol)	M	Induced: MDR pumps Repressed: hypha-specific genes	Limited changes	37
Prostaglandin E_2	M	Induced: no significant pattern Repressed: protein synthesis, metal transport	Responses are very transient	123
In vivo experiments				
Kidney (mouse)	M	Repressed: aspects of transcription, translation, cell cycle progression	Most regulated genes were repressed	9
Kidney (rabbit)	M	Induced: adhesion, glyoxylate cycle/ alternative carbon metabolism, MDR pumps Repressed: protein synthesis, others	In vivo cells compared to broth-grown controls	224
Peritoneum	M	Induced: glycolysis, heat shock, iron uptake, hypha-specific genes	SC5314 compared to a noninvasive strain	217
Catheter biofilm	M	Induced: protein synthesis Repressed: cell cycle progression	In vivo cells compared to RPMI-grown controls	154
Oral cavity or gastrointestinal tract (piglets)	M	Induced: hypha-specific genes, EFH1, SOD3	In vivo cells compared to broth-grown controls	226

[a]All data from *C. albicans* except where indicated. OSR, oxidative stress response; MDR, multidrug resistance; GO, gene ontology.
[b]Major responses are reported by the authors.

and its ability to undergo the yeast-hypha transition inside macrophages has been known for 50 years (210). Continued hyphal elongation eventually distends the macrophage membrane to the point of rupture (Color Plate 7). If immortalized cell lines are used, such as the J774A.1 or RAW264.7 line, filamentous growth is a ubiquitous response, occurring in nearly every fungal cell. In primary cells with greater antifungal activity, germ tube formation is less common but is still seen in many cells. In the body, macrophages can be seen in association with germ tubes and hyphae, but it is hard to determine whether the filaments are formed before or after the initial contact with the macrophage.

Why *C. albicans* forms filaments inside macrophages is not known. The yeast-to-hypha transition is stimulated by multiple conditions, including physiological temperature, neutral-alkaline pH, nitrogen starvation, serum, CO_2, or a variety of chemical inducers, such as *N*-acetylglucosamine, some amino acids, and alcohols. The specifics of morphogenesis are beyond the scope of this chapter but have been extensively detailed in the previous edition of this book (23, 24). Inducing conditions exist outside of the macrophages in coculture experiments (serum, 37°C, and neutral pH), but only the temperature is clearly the same inside the macrophage. Avid filamentation occurs regardless of whether the experiments are performed in a CO_2-rich environment (M. Lorenz, unpublished observations), though it has recently been suggested that *C. albicans* can produce sufficient CO_2 to induce germ tube formation (84).

C. albicans germinates within macrophages far more avidly than other species, though germ tubes can be seen at a lower frequency in *C. tropicalis* (18, 210). Even the closely related *C. dubliniensis* does not form hyphae after phagocytosis (140). Interestingly, Moran et al. deleted the *C. dubliniensis* homolog of *NRG1*, a global transcriptional regulator that represses hyphal formation in *C. albicans* (22, 142), and found that the mutants were hyperfilamentous, germinated in macrophages, and caused increased damage to reconstituted epithelial layers. They were, nevertheless, no more virulent than wild-type *C. dubliniensis* in a systemic murine model (140). *C. glabrata* appears to form pseudohyphae only in response to nitrogen starvation (26) and does not undergo morphogenesis in the macrophage. Data for other species are sparse.

Conventional wisdom suggests that germination within the macrophages promotes fungal survival by destroying the phagocyte and freeing the hyphae. While undoubtedly true, even cells locked in the yeast form survive well, at least in macrophage-like cell lines. One means to generate a nonfilamentous strain is to simultaneously delete the CPH1 and EFG1 transcription factors (125); this strain survives indefinitely inside macrophages, even beginning to replicate within a few hours (130; M. Lorenz and S. Vylkova, unpublished observations). Overexpression of *NRG1* also inhibits morphogenesis, and while there are no published data regarding macrophage survival, cells restricted to the yeast form persist asymptomatically in tissues for extended times and can initiate a lethal infection if the hyphal inhibition is relieved (193), suggesting that these cells effectively resist destruction by immune cells in vivo as well.

In stark contrast, *C. albicans* cells ingested by neutrophils do not germinate (188). Experiments with neutrophils are generally done with primary cells isolated from peripheral blood, due to both the ease of preparing such cells and the lack of good neutrophil-like cell lines. The lack of filamentation cannot be entirely ascribed to the greater antifungal activity of these neutrophils, as primary macrophages also have potent antifungal activity yet do not prevent filamentation. As described above, these experiments use conditions (physiological temperature, pH, and CO_2) that should stimulate germ tube formation; whether neutrophils specifically inhibit filamentation or macrophages induce it is an open question.

Endothelial and epithelial cells also induce germination in *C. albicans* (163). True hyphae bind most tightly to both cell types, whereas pseudohyphae or yeasts are more weakly associated (166). This is at least partly due to the activity of the hypha-specific adhesin ALS3 (see below). An interaction between ALS3 and host cell cadherins mediates endocytosis, and continued hyphal growth within the endothelial or epithelial cells disrupts the integrity of these monolayers (67, 164, 166–168, 186). Filamentation is essential for damage to the host cells, as nonfilamentous cells such as the *efg1*Δ/Δ *cph1*Δ/Δ mutant are endocytosed, albeit at a lower rate than hyphae, but cause no damage (166). However, *C. albicans* can pass through brain microvascular endothelial cells via an endocytic mechanism without damaging these cells (104).

STRESS RESPONSES

The interaction of microbes with phagocytes is clearly stressful—the intent, after all, is to kill the invading cells. The best-understood component of these antimicrobial activities, from both the host and pathogen perspectives, is the respiratory burst of ROS and RNS. The signaling pathways that govern these responses are described in detail elsewhere in this volume, so this chapter focuses on the effectors themselves and their roles in mediating the interaction with host cells.

ROS and RNS

Pathogens face ROS from both endogenous and exogenous sources. The primary endogenous source is superoxide generated as a side effect of mitochondrial electron transport during aerobic respiration. Detoxification of endogenous ROS is a challenge shared by all aerobic cells, though it is particularly problematic in mammalian pathogens since generation of superoxide is generally greater at 37°C than at cooler temperatures. Fungi have both enzymatic and nonenzymatic antioxidant mechanisms, and these have been repeatedly shown to be critical during contact with host cells (reviewed in reference 33).

Mammalian pathogens must particularly defend against extracellular sources of ROS, primarily from the respiratory burst of phagocytes. A highly effective means for overcoming this stress would be to detoxify these oxidants outside the cell, and in fact, *Candida* species have evolved mechanisms to do so. Several antioxidant proteins that are exclusively intracellular in *S. cerevisiae* have been found on the cell wall in *C. albicans*, including catalases and thioredoxin (TRX) peroxidases (43, 219, 220), as well as a unique family of secreted, GPI-anchored superoxide dismutases (SODs [25]) that detoxify ROS produced by phagocytes (70, 74, 136). Specific examples of extracellular antioxidants are highlighted below and in Table 2.

SODs

SODs convert the superoxide anion into hydrogen peroxide, which can then be converted to water by catalases. All fungi studied to date have at least two SODs that, despite similar enzymatic activities, are unrelated: a mitochondrial

TABLE 2 Antioxidant proteins located on the C. *albicans* cell surface

Protein	Function	GPI anchor	Localization confirmed	Reference(s)
SOD4	SOD	Yes	Yes	46
SOD5	SOD	Yes	Yes	70
SOD6	SOD	Yes	No	74
CAT1	Catalase	No	Yes	43
TSA1	Thiol-specific antioxidant	No	Yes	43, 220

enzyme using manganese as the cofactor (MnSOD) and a copper/zinc enzyme (Cu/ZnSOD) that is primarily cytoplasmic but has also been found in the mitochondria (73). *S. cerevisiae* has one of each, SOD1 (Cu/Zn) and SOD2 (Mn), and deletions of either confer a variety of phenotypes related to oxidative damage, including sensitivity to exogenous ROS, high mutation rates, inability to grow under highly aerobic conditions, defects in amino acid biosynthesis, and rapid loss of viability in stationary phase (124, 128, 205, 221). Surprisingly, these genes are not synthetically lethal, but phenotypes are worsened in the double mutant, indicating at least some redundancy in the pair (124).

Notably, the *SOD* gene families are expanded in all pathogenic *Candida* species with complete genome sequence available, except for C. *glabrata*, which is more closely related to *S. cerevisiae* (25). C. *albicans* has six SODs: four Cu/Zn enzymes and two MnSODs. SOD1 is the best characterized of these; it is highly conserved, bearing 69% identity to the Cu/Zn SOD1 of *S. cerevisiae* and a remarkable 57% identity to bovine SOD (98). The phenotypes of *sod1*Δ/Δ mutant strains are milder than for its *S. cerevisiae* counterpart, presumably due to the greater redundancy in C. *albicans*: a moderate sensitivity to menadione (but not hydrogen peroxide), a partial lysine auxotrophy, and defective hyphal growth. Importantly, the *sod1*Δ/Δ mutant was more susceptible to killing by macrophages and was slightly reduced in virulence in a systemic mouse model (99).

The mitochondrial SOD2 also shows high similarity to other MnSODs (180). Sensitivity to oxidative stresses is more pronounced in the *sod2*/Δ/Δ strain than the *sod1*Δ/Δ one, and this strain is unable to grow under hyperoxic (100% O_2) conditions (97). The *sod2*Δ/Δ mutant is somewhat more susceptible to killing by neutrophils than wild-type cells (34), but it is not attenuated in the mouse intravenous challenge model (97). SOD3 is an atypical MnSOD; it is predicted to be cytosolic and is most strongly expressed in stationary-phase cells regardless of oxidative stress (119). This fits with data from *S. cerevisiae* showing that cells lacking either *SOD1* or *SOD2* rapidly lose viability in stationary phase (128, 129), but this has not been directly tested in C. *albicans*, nor has the role for this protein in phagocyte interactions or virulence been assessed.

Most unusually, all the pathogenic CUG *Candida* species have Cu/ZnSODs predicted to be extracellular and GPI anchored (25). C. *albicans* has three, SOD4 to -6, and cell wall localization has been confirmed for SOD4 and -5 (46, 70). *SOD5* is induced by neutrophil contact, oxidants, and nonfermentable carbon sources and is expressed more highly in hyphae (70, 136), as is *SOD4* (74). Frohner and colleagues studied the strains by deleting for the SODs in an in vitro coculture system with primary bone marrow-derived macrophages and found that dramatically higher levels of extracellular ROS were present when *sod5*Δ/Δ mutants were used than with the wild type. This was further elevated in a *sod4*Δ/Δ *sod5*Δ/Δ double mutant, while *SOD1* to *-3* and *SOD6* had no effect (74). They proposed that the function of SOD4 and SOD5 is to degrade ROS produced during the respiratory burst of phagocytes. Not surprisingly, *sod5*Δ/Δ mutants were more than twice as susceptible to killing by bone marrow-derived macrophages, and the *sod4*Δ/Δ *sod5*Δ/Δ strain was almost 10-fold reduced in viability in this model (74). SOD5 is also required for full virulence in the systemic mouse model (136).

Taken together, these reports emphasize the importance of the SOD family during host cell contact and during pathogenesis in C. *albicans*. The large number of SODs, relative to other fungi, and the partial redundancy suggest that developing a robust resistance to oxidative stresses, including from exogenous sources, was critical during the evolution of *Candida*. SODs have not been studied at a molecular level in any of these species other than C. *albicans*, so defining the exact roles of these enzymes in pathogenesis requires further investigation.

Catalases

Catalases convert hydrogen peroxide into water and oxygen and thus complete the detoxification of superoxide. C. *albicans* has a single predicted catalase, CAT1 (which has also been published as CTA1 and CCT1), in contrast to *S. cerevisiae*, which has two (cytosolic CTT1 and peroxisomal CTA1). The C. *albicans* protein was localized to the peroxisome by immunoelectron microscopy, and this localization was dependent on the PTS1 receptor PEX5 (170), though CAT1 does not have a classical C-terminal PTS1 motif. A subsequent study showed a substantial amount of catalase to be in the soluble, rather than organellar, fraction of cell lysates (169). Catalase has also been identified in cell wall fractions as a high-affinity binding protein for mammalian plasminogen (43). It seems likely that catalase is distributed across multiple cellular compartments, including, potentially, the cell wall.

*cat1*Δ/Δ mutants are hypersensitive to oxidative stress and killing by neutrophils and are attenuated in a systemic mouse model (148, 227). Peroxide stimulates hyphal morphogenesis in a CAT1-dependent manner (147, 150). The mutant has some unexpected pleiotropic phenotypes, including an increased sensitivity to detergents and elevated temperature (147, 150). *CAT1* is strongly induced at the mRNA and protein levels by oxidative stress in vitro and in contact with both macrophages and neutrophils (62, 70, 117, 130, 148, 188). It is also induced in a small percentage of cells recovered from mouse kidney (61).

Candida glabrata is extremely resistant to oxidative stresses and is able to grow at hydrogen peroxide concentrations up to 20-fold higher than tolerated by *S. cerevisiae* and 2- to 3-fold higher than C. *albicans* (44). C. *glabrata* also has

a single catalase gene, *CTA1*, which is strongly upregulated by phagocytosis (183). Disruptants are much more sensitive to peroxide stress; surprisingly, however, *cta1*Δ mutants are unaffected in persistence in internal organs in a mouse systemic model (44). CTA1 has a PTS1 motif at the C terminus and is thus likely to be peroxisomal, though this has not been tested and does not preclude localization to other cellular compartments. Catalases have not been studied in other *Candida* species.

Thioredoxin

The TRX antioxidant system reduces protein thiol bonds through a chain of electron transfers, from NADPH to TRX reductase (TRR) to TRX to the protein target. Along with the similar glutathione system, TRXs are critical in antioxidant responses and in maintaining redox balance (reviewed in reference 88). Many of the *C. albicans* components are induced by oxidative stress, phagocyte contact, or both. *C. albicans* has three TRX homologs (TRX1, TRX2, and TXL1) and a single TRR (TRR1); *TRX1* and *TRR1* are induced by oxidative stress and neutrophil contact (62, 70, 220) but not by macrophages (65, 130). There are no expression data for the other genes, and none have been knocked out.

An associated protein, the thiol-specific antioxidant (TSA1), a TRX peroxidase, is induced by hydrogen peroxide and by neutrophil contact (70, 188, 220). TSA1 is mostly nuclear in oxidant-exposed yeast cells but is found in the cell wall of hyphae (204, 219, 220), where it apparently can bind mammalian plasminogen (43). Deletion of TSA1 confers sensitivity to hydrogen peroxide, dithiothreitol, and cell wall-perturbing agents such as Congo red, and hyphal growth is abnormal in *tsa1*Δ/Δ cells under some conditions (204, 220). Nevertheless, TSA1 is not required for systemic virulence (220) and has not been directly tested in phagocytosis models. The current genome annotation suggests that the TSA1 gene is a duplicated gene: a nearly identical open reading frame (ORF) is located about 40 kb from the TSA1 gene, opening the possibility of redundancy.

Glutathione

The glutathione system is analogous to TRX, with glutaredoxins (GRXs), glutathione reductases (GLRs), and glutathione peroxidases (GPXs). Added to this is the tripeptide glutathione, which mediates electron transfer between GLRs and GRXs (reviewed in reference 88). In *S. cerevisiae*, the glutathione and TRX systems are partially redundant and at least one is required for viability (54). There have been limited molecular studies with *Candida*: only GRX2 (also known as TTR1), one of four predicted GRXs, has been characterized. The corresponding gene is induced by neutrophils and hydrogen peroxide but not macrophages (62, 70, 130, 188). Mutant strains are impaired for survival in neutrophils and in a disseminated mouse model (34). The sole GLR (*GLR1*) is similarly induced in response to oxidative stress in vitro (2) and by neutrophils (70), but it has not been studied further. There are also four genes encoding predicted GPXs, including three in a cluster on chromosome 1; *GPX1* is neutrophil induced (70).

Nonenzymatic Antioxidants

Fungal cells produce the storage carbohydrates glycogen and trehalose under nutrient-replete conditions, and a role for these in oxidative stress resistance has long been appreciated (reviewed in reference 10). The synthesis and function of trehalose have been investigated in *C. albicans* in some detail. Trehalose is a disaccharide of two glucose molecules in an α1-1 linkage, produced in sequential steps by the TPS1 and TPS2 enzymes. *TPS1*, encoding trehalose-6-phosphate synthase, is essential for growth in *S. cerevisiae* on glucose (85). A *C. albicans tps1*Δ/Δ strain grows normally at 30°C but is unable to utilize glucose at elevated temperatures, while the *tps2*Δ/Δ mutant has a reduced growth rate in glucose above 37°C (230, 231). Both strains are sensitive to oxidative stresses, have perturbations in cell wall function, are susceptible to killing by macrophages, and are significantly attenuated in virulence in the disseminated mouse model (8, 134, 137, 138, 231). Trehalose synthesis is induced by oxidative stresses (27), but *TPS1* and *TPS2* are not significantly induced in microarray analyses of in vitro oxidative stress or macrophage/neutrophil phagocytosis (62, 70, 130). Thus, trehalose is a key mediator of resistance to oxidative and other stresses, plays a role in cell wall structure, and is critical for virulence in both in vitro and in vivo models.

Regulation of Antioxidant Responses

Many of the genes described above are induced in the presence of oxidative stresses, phagocytes, or both. There are two main inputs to this regulation: the HOG1 mitogen-activated protein (MAP) kinase pathway and the CAP1 transcription factor. CAP1, the homolog of *S. cerevisiae* YAP1, confers resistance to oxidative stresses and to some antifungal drugs (2), and *cap1*Δ/Δ mutants are sensitive to neutrophil killing (70). CAP1 directly regulates many of the genes described above, including *CAT1*, *GLR1*, *TRX1*, and *SOD1* (2, 63, 240), and is required for trehalose accumulation in response to oxidative stress (27). CAP1 activity is regulated by shuttling between the nucleus and cytoplasm, possibly as a result of direct modification by oxidants (232).

The HOG1 MAP kinase pathway responds to multiple stresses, primarily oxidative and osmotic (reviewed in referenc 33), and the *hog1*Δ/Δ deletion mutant is sensitive to both (6, 7). The transcriptional targets of HOG1 overlap with CAP1, but they appear to be at least partially independent (7, 63). In response to oxidative challenge, HOG1 is activated by a phosphotransfer system similar to prokaryotic two-component systems, and mutations of two of the constituent proteins, SLN1 and SSK1, both confer sensitivity to oxidative stress. The *ssk1*Δ/Δ mutant, in particular, is very susceptible to phagocyte killing and is avirulent (32, 55, 144). HOG1 is phosphorylated and translocated to the nucleus in response to stress (206), but what is downstream of HOG1 is not clear.

These pathways are only the tip of the iceberg for antioxidant regulation, however. *SOD5* is also regulated by the RIM101 alkaline sensing pathway and is not induced in a nonfilamentous *cph1 efg1* mutant, even in the presence of stress (15, 136, 149). The *C. glabrata* catalase is controlled by at least four transcription factors, YAP1, SKN7, MSN2, and MSN4, and how they work together is not known (44). Much work remains to understand the transcriptional networks that regulate oxidative stresses in these pathogens.

Response to RNS

In addition to ROS, mammalian phagocytes produce nitric oxide (NO), which can be oxidized into a variety of RNS. *C. albicans* is sensitive to NO at low millimolar concentrations, and NO provokes a focused transcriptional response—only nine genes are upregulated for the duration of exposure (94). One of these, *YHB1*, encodes a homolog of NO-detoxifying flavohemoglobins from other species. Deletion of *YHB1* confers increased sensitivity to NO, and *yhb1*Δ/Δ

mutants are modestly attenuated in a mouse model (94, 218). This attenuation, surprisingly, is still seen in a knockout mouse strain lacking the inducible NO synthase (94). There are two additional flavohemoglobin homologs in C. *albicans*, YHB4 and YHB5. Mutant strains lacking either of these have no obvious phenotypes (218), but their presence does open the possibility of redundancy with YHB1 in vitro or in vivo. NO induction of YHB1 is mediated by the CTA4 transcription factor (39), but the mechanism for activation by NO is not known. None of these have been tested directly in in vitro coculture models, nor have homologs been studied in any other pathogenic C*andida* species.

Summary: Oxidative and Nitrosative Stresses

The respiratory burst of neutrophils and macrophages is an important component of the antimicrobial activities of these cells, and C. *albicans* has evolved numerous routes to overcome these stresses (see the model in Fig. 1). Importantly, a few of these antioxidants are secreted, initiating detoxification before intracellular damage can occur. In general, these systems are induced by oxidative stresses and by neutrophil contact. Some are also induced by macrophages, though many experiments used either monocytes or cell lines with lower antifungal activity potentially due to smaller respiratory bursts. Mutations in some genes have been tested in coculture and in vivo, and with several exceptions, these systems are important contributors to fungal survival and virulence in the mammalian host. Further work is obviously needed to fully understand when and where oxidative stress is greatest and to understand the regulation of these pathways.

NUTRIENT STARVATION

It has long been appreciated that some auxotrophic mutants are much less virulent in animal models; the *ura3* and *ade2* mutants are notable examples and are required for infectivity in multiple fungal species, including C. *albicans*, in both

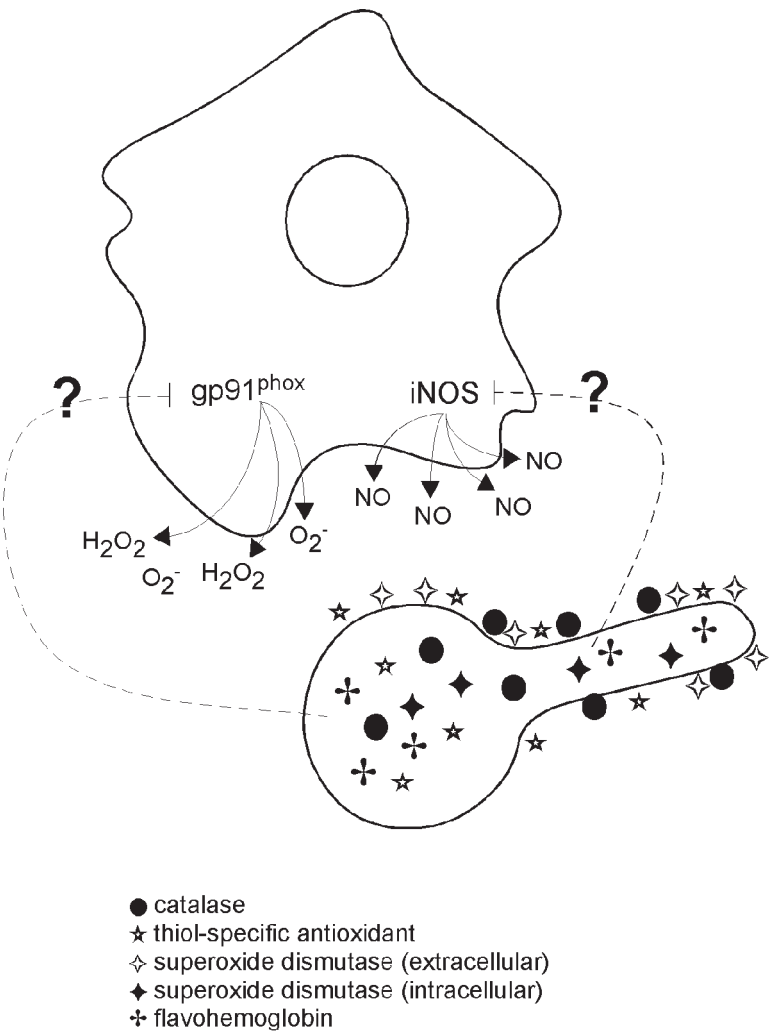


FIGURE 1 Extracellular detoxification of ROS. Depicted are a generic phagocyte producing superoxide, hydrogen peroxide, and NO, opposed with a C. *albicans* cell elaborating cell surface and intracellular antioxidant mechanisms (see symbol key). 10.1128/9781555817176.ch17f1

systemic and oral models (20, 40, 53, 112, 120). Inositol synthesis is also essential in vivo in both *C. albicans* and *C. glabrata*, highlighting the importance of nucleotide availability (17, 35). This has posed a problem in the almost ubiquitous (until recently) use of *URA3* as a gene deletion marker; the magnitude of this problem became apparent during the analysis of the *HWP1* (hyphal wall protein) gene, in which the chromosomal position of *URA3* was found to strongly influence several phenotypes, including virulence (202, 214). Despite these findings, there has not been a systematic attempt to catalog the effects of auxotrophic mutations on virulence.

Carbon Starvation

A surprising finding from the analysis of macrophage-phagocytosed cells was the role of carbon starvation, which represented nearly two-thirds of the genes whose expression changed (130, 131). Induced pathways included gluconeogenesis, the glyoxylate cycle, and fatty acid catabolism, along with many nutrient transporters. Concurrent repression of protein synthesis and glycolysis made the response to phagocytosis remarkably similar to the typical fungal starvation response, as seen in *S. cerevisiae* (81). Subsequent microarray analysis confirmed the extensive coregulation of genes during macrophage phagocytosis and glucose starvation (130). Using similar coculture systems, the same profile has been observed by differential display (173), in monocytes (70), and by proteomic technologies (65). Only a very limited carbon starvation profile is seen in cells phagocytosed by neutrophils, though these cells upregulate other nutrient pathways (70, 188). Using single-cell, green fluorescent protein (GFP)-based reporter assays, induction of several genes, including those in the glyoxylate cycle (*ICL1*) and gluconeogenesis (*PCK1*), were found to be upregulated both in cell culture and in kidney lesions (12), showing that carbon limitation is relevant in vivo as well. Microarray analysis of cells recovered from animals also confirms the relevance of alternative carbon metabolism (9, 224).

A number of these pathways have been directly tested for virulence. Strains mutated for the glyoxylate cycle (*ICL1*) and gluconeogenesis (*PCK1* and *FBP1*) show substantial attenuation in the disseminated mouse model (12, 131, 174). Utilization of fatty acids via β-oxidation is relatively less important, with mutants in the catabolic enzyme FOX2 or the regulator CTF1 only modestly attenuated (170). Other processes, such as acetyl coenzyme A metabolism and transport, appear to be dispensable during infection (28, 212, 237). Nevertheless, there is solid evidence that *C. albicans* makes use of nonsugar compounds as important nutrients in vivo. Despite this, only a few of these mutants have been tested in coculture models with phagocytes, and none have shown obvious defects (Lorenz, unpublished).

Amino Acid Starvation

Cells phagocytosed by neutrophils induce several amino acid biosynthetic pathways—arginine, methionine, cysteine, and lysine (70, 71, 188). As for carbon metabolic pathways, there is not a comprehensive understanding of which amino acids are limiting in vivo. There has been a more systematic attempt to assess virulence of amino acid auxotrophic mutants, albeit generally to identify appropriate gene disruption markers. *C. albicans* strains that are auxotrophic for histidine (*his1*Δ/Δ), leucine (*leu2*Δ/Δ), or arginine (*arg4*Δ/Δ) all retain full virulence in a disseminated mouse model (6, 112, 158). In *C. glabrata*, a project with similar goals has shown that strains auxotrophic for histidine, leucine, and tryptophan are all fully virulent (103). While multiple auxotrophies affect virulence in some fungal pathogens, notably *Cryptococcus* (108, 110, 111, 152, 165, 228), in *C. albicans* only *ILV2*, required for isoleucine, leucine, and valine synthesis, has been shown to be required for virulence (109). Other auxotrophic mutants have not been tested in animal or cell culture models, so the spectrum of avirulent auxotrophies in *Candida* species is likely to be broader than it currently appears.

The arginine biosynthetic pathway, though apparently not required in vivo, does affect the interaction of *C. albicans* with macrophages. The *ARG* genes are strongly upregulated in both macrophage- and neutrophil-phagocytosed cells (70, 130, 188); this is the only amino acid pathway significantly induced by macrophages (130). It has been suggested that the purpose of this induction might be to produce arginine, not for protein synthesis but to break it down: arginine is degraded by two sequential steps catalyzed by arginase (CAR1) and urea amidolyase (DUR1,2) into citrulline, urea, and CO_2, the last of which is a potent stimulator of hyphal morphogenesis (113). Mutants in *DUR1,2* are impaired in germ tube formation in a macrophage coculture system (84), so there is some evidence for this model.

Autophagy

The nutrient deprivation that apparently accompanies growth in vivo induces many systems for nutrient acquisition and utilization. One ready source for nutrients is the breakdown of existing cellular macromolecules, and proteasome subunits are induced following macrophage phagocytosis (65). Another important scavenging mechanism is autophagy, in which cellular components are enclosed in a membrane-bound vesicle and degraded (reviewed in reference 177). Autophagy is required for virulence in *C. glabrata*, where deletions of ATG11 or ATG17 both impaired long-term survival in macrophages (183), and is also important in other fungal pathogens, for instance, *Cryptococcus neoformans* (161). Surprisingly, however, *C. albicans atg9*Δ/Δ mutant cells, which are defective in autophagy and sensitive to nitrogen starvation, remain fully virulent and unaffected during coculture with macrophages (162), despite the induction of *ATG9*, and other autophagy-related genes (130).

Summary: Nutrient Starvation

The combined data presented above clearly indicate that *Candida* cells perceive the environment within the phagocyte and in certain host niches to be nutrient poor. A surprise was the finding that the nature of this starvation is different between different phagocytes. Some, but by no means all, of these metabolic pathways are required for full virulence, and we are beginning to develop a better picture of "life on the inside" in terms of basic cellular needs. This is both biologically interesting and potentially important from a therapeutic sense, as many of these pathways do not have mammalian homologs. In fact, of the 228 genes found in all sequenced fungi but not in any mammal (as of 2005), the vast majority of them are involved in nutrient uptake and amino acid biosynthetic pathways (149). If shown to be necessary for virulence, these pathogen-specific enzymes would be ideal *Candida*tes for drug development.

MODULATION OF IMMUNE FUNCTION

A picture emerges from the above sections of a phagocytosed *Candida* cell racing to respond to various cytotoxic

stresses generated by the immune system, and at some level, this is indeed the case. However, it is increasingly clear that *C. albicans*, at least, is not a passive victim in this interaction—that it actively modulates the function of innate immune cells. This is not a new idea: it was suggested more than three decades ago that *C. albicans* could suppress lymphocyte maturation and function (184, 185) and that even fixed cells manifested this activity (182). Later, extracted components of the *C. albicans* cell wall were found to inhibit phagocytosis by macrophages (215). Recently, a variety of ways in which *C. albicans* alters immune cell function have been identified; for the most part these observations are not understood either at a molecular level or in terms of how they might contribute to virulence in vivo, and they represent an exciting area for future research.

Secreted Hydrolase Activity

A common feature of the CUG *Candida* species is the presence of expanded families of secreted hydrolytic enzymes, notably proteases and lipases (25). Biochemical studies have shown that some of these proteins have activity against substrates relevant to the immune response, but genetic evidence that they are required for infectivity has been hampered by their redundancy: *C. albicans* has 10 secreted aspartyl proteases (SAPs) and 10 lipases alone. Nevertheless, the accumulated evidence strongly supports a key role for these enzymes in immune evasion (reviewed more completely in reference 195).

Extracellular Proteases

The SAP family has been the subject of extensive study in *C. albicans*, and a full discussion is beyond the scope of this chapter. Readers interested in more detail are encouraged to consult two extensive reviews on the subject (145, 195). The SAPs—which are unique to the CUG *Candida* species—were always considered to be virulence factors, but a definitive demonstration of this was for years complicated by their redundancy. In general, they fall into three groupings: SAP1 to -3 are more important in mucosal infections, SAP4 to -6 are important in systemic infections, and SAP9 and -10 are GPI-anchored cell wall proteins that appear to be particularly important during phagocyte contact. SAP7 and -8 have not been extensively studied. The SAPs have been shown to degrade a large number of relevant host substrates, including immunoglobulins, complement, and extracellular matrix, and individual SAP genes are differentially expressed in both in vitro and in vivo infection models and in clinical samples from from human patients (summarized in reference 145). Here a few salient points regarding SAP involvement in host cell interactions are summarized.

SAP1 to -3 are primarily associated with mucosal infections, and a *sap1-3*Δ triple mutant strain has been shown to cause less epithelial damage and elicit a smaller cytokine burst than wild-type cells (194), although a more recent study has found no difference between *sap1-3*Δ and the wild type in an in vitro reconstituted epithelial model (122). In vitro, SAP2 is uniquely required for growth on protein as a nitrogen source (95).

SAP4 to -6 are preferentially expressed in hyphae and are seen to be more important in disseminated models. Interestingly, the mRNA for these genes is concentrated at the hyphal tip, though it has not been determined whether this leads to localized protein secretion (59). These genes are strongly induced by macrophage phagocytosis, and a *sap4-6*Δ strain is more susceptible to killing by macrophages and is highly attenuated in a guinea pig infection model (19, 191).

SAP9 and -10 are cell surface, glycosylated, and GPI-anchored proteases that share similarity to the yapsin protease family in *S. cerevisiae* and *C. glabrata* (4). These proteins contribute to virulence in reconstituted epithelial models, promote binding to epithelial cells, and are important for complete cell separation following cytokinesis (4). Curiously, *sap9*Δ/Δ mutants induce *less* ROS and survive better in the presence of neutrophils than wild-type strains, perhaps indicating that phagocytes specifically recognize these cell surface SAPs (89). In contrast, the role of the 11-member *C. glabrata* yapsin protease family in virulence is quite clear, facilitated in part by their genomic organization, with eight of the genes in a single cluster. Several of these genes are upregulated upon phagocytosis, and a complete, 11-gene-knockout strain (*yps1-11*Δ) is significantly impaired in survival in macrophages and in vivo (106).

Lipases and Phospholipases

The 10 *C. albicans* lipase genes were first identified in a *Saccharomyces*-based screen for extracellular lipase activity, and it was quickly realized that there was a multigene family (75, 96). Like the SAPs, they are expressed in systemic and oral infections (211). The importance of lipases in virulence was established, unusually, in *C. parapsilosis*: a mutant with a knockout of *LIP1* and *LIP2*, which are adjacent in the genome, is strongly impaired in macrophage survival and in a systemic mouse model (80). Subsequently, a *C. albicans* strain lacking *LIP8* was shown to be attenuated in vivo (79).

C. albicans also encodes phospholipase A, B, C, and D activities, most of which are expressed in samples recovered from infected patients (146). With the exception of the essential PLC1 (115, 116), all of these are predicted to be secreted or on the cell surface (25). Two of the four PLB homologs are predicted to have GPI anchors, a modification not found in *S. cerevisiae*. PLB1, PLB5 (a phospholipase A), and PLD1 have been implicated in systemic virulence (51, 121, 216). PLB1, in particular, is expressed in vivo, and *plb1*Δ/Δ mutants are highly attenuated and less able to invade endothelial cells (121).

Suppression of ROS and RNS Function

As described above, *Candida* species have a vigorous defense against oxidative and nitrosative stresses, and this is important in vivo. Better still would be to prevent the production or accumulation of these free radicals, and indeed, it appears that *C. albicans* has some ability to do just that. *C. albicans* cells elicited far less ROS from either primary or cultured macrophages or neutrophils (from mice or humans) relative to heat- or UV-killed cells. Importantly, this was not mediated by cell wall components—both killed cells and isolated walls stimulated ROS production, but intact live cells reduced it (225). Similarly, there is evidence that live *C. albicans* cells inhibit the release of nitric oxide from macrophages as well (38, 64, 200). The nature of this suppressive activity is unknown. Combined with the detoxification systems, the ability to reduce the amount of ROS or RNS from phagocytic cells, even by a modest amount, may go a long way towards blunting this aspect of the immune response.

Interference with Intracellular Trafficking

A "model" microbe is ingested into the phagosome, which then undergoes an intricate series of events eventually fusing with acidic lysosomal compartments, while gaining and losing membrane proteins that serve as useful markers of the process. A number of pathogens derail phagosomal maturation process as a means of intracellular survival;

Mycobacterium tuberculosis, for instance, inhibits phagosome-lysosome fusion, blocking acidification and the acquisition of lysosomal degradative enzymes (reviewed in reference 222). In contrast, there are conflicting reports on the intracellular fate of *C. albicans*. It was found to not colocalize with acidophilic dyes such as acridine orange or LysoTracker, evidence against lysosomal fusion (135, 139). The alkaline-induced PRA1 (pH-regulated antigen) is expressed in macrophages, further evidence that *C. albicans* is in an unfused phagosomal compartment (135). Later, *C. albicans* was shown to be rapidly recruited into compartments that stained with the lysosomal membrane protein LAMP-1, evidence that fusion had occurred (105), although LAMP-1 is not an unambiguous marker. Phagosome-lysosome fusion was found to be dependent on the assay conditions, perhaps explaining the discrepancies—macrophages bound to a collagen matrix showed high levels of fusion after phagocytosis of live *C. albicans*, but unbound macrophages did not. In both cases, killed cells were always found in the phagolysosome (155). Most recently, a careful study with multiple endocytic markers, wild-type, nonfilamentous mutants, and heat-killed *C. albicans* cells demonstrated that trafficking of live *C. albicans* cells is indeed aberrant (64). At least some *C. albicans* cells end up in a vesicle contiguous with the endoplasmic reticulum, though the marker studies did not conclusively identify the compartment; this fate is more common in the wild-type strain than in the nonfilamentous *cph1 efg1* mutant. The mechanisms behind this alteration in endocytic trafficking are yet unknown.

Effects on Binding and Chemotaxis of Phagocytes

To phagocytose a microbe, the innate immune cells must sense its presence, chemotax toward it, and bind it tightly, and several *Candida* species are able to modulate these processes. *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. parapsilosis*, and *C. glabrata* all release a chemoattractant(s) that stimulates directed movement of neutrophils towards the fungal cell (21, 45, 57, 83). At least one of these chemoattractants signals through the formylmethionyl receptor on neutrophils, and preliminary characterization is consistent with a small peptide (57, 83), though other reports have suggested that chemotaxis can also be stimulated by β-glucan moeities (192). Interestingly, the presumptive peptide signal is regulated by the white-opaque phase variation—opaque cells do not produce it (83). Opaque cells are also more poorly phagocytosed by RAW264 and *Drosophila* S2 cells (127).

Another important receptor mediating phagocyte chemotaxis and adhesion is the CD11b/CD18 (CR3) $\alpha_M\beta_2$ integrin (68, 69). The major *C. albicans* ligand for this integrin is PRA1, a cell surface protein that also binds fibronectin (30, 201, 207). PRA1 is also a major component of the ability of *C. albicans* to bind several complement factors, including plasminogen, factor H, and FHL-1, which are apparently co-opted and activated on the fungal cell surface to degrade extracellular matrix and complement (133, 171). As mentioned above, the CAT1 catalase also binds plasminogen (43), so there are multiple binding sites for this important protease. Thus, PRA1 appears to have competing effects on *Candida*-phagocyte interactions, and its overall contribution to virulence has not been reported.

Apoptosis

Hyphal morphogenesis can kill macrophages by mechanical disruption, but *C. albicans* apparently triggers apoptosis in several cell types. Incubation of macrophages with either whole cells or isolated cell wall phospholipomannan leads to the appearance of several classical markers of apoptosis, accompanied by reduction in both Bad phosphorylation and Bcl-2 protein levels (100). Neutrophils also undergo apoptosis at an elevated rate after contacting *C. albicans*, though coculture supernatants inhibit apoptosis in naïve cells (187). The quorum-sensing molecule farnesol also induced apoptotic-like death in macrophages, squamous carcinoma cells, and human sperm cells (1, 179, 199) and altered cytokine production by macrophages, promoting a tumor necrosis factor alpha proapoptotic profile (151). In other cell types, however, there is evidence that *C. albicans* inhibits apoptosis, including in vascular endothelial cells and a monocyte cell line (13, 87). Thus, *C. albicans* interferes with phagocyte function via modulating apoptosis, partly via secreted compounds. A role for this in vivo is supported by an increase in apoptosis of hepatocytes in *C. albicans* infected animals (178), but this obviously requires much more study.

ADHESION AND INVASION

The adhesion between *Candida* cells and phagocytes is primarily driven by receptors on the mammalian cell that recognize carbohydrate moieties on the cell wall, though there are some fungal proteins that mediate adhesion as well. Adhesion is also a central feature of the interaction of *Candida* with endothelial and epithelial cells, but binding is mostly due to the actions of fungal, and not mammalian, proteins. As we discuss below, there has been a notable expansion of putative adhesions—cell surface glycoproteins with and without GPI anchors—in the pathogenic *Candida* species. As for the SAPs and lipases, a full understanding of the role of these proteins has been complicated by their sheer number. The *C. albicans* genome encodes a remarkable 115 predicted GPI-anchored proteins (181). Several earlier publications had different estimates, though in the same range, obtained using older genome assemblies and/or predictive algorithms (47, 58, 213). The similarly sized yeast genome encodes an estimated 58 such proteins (29), and the vastly larger human genome encodes only 188 predicted GPI-anchored proteins (http://mendel.imp.ac.at/gpi/Hs/hs.html).

Adherence roughly correlates with virulence in both in vitro and in vivo models. In a model of skin infection, *C. albicans* adhered most tightly and caused the most tissue damage; *C. tropicalis* was weaker at both, and the other tested species adhered poorly and did not cause any notable tissue damage (176). Adherence and virulence also correlate in reconstituted epithelial models (16, 49, 189, 239), though this is not absolute (234). Since adherence of *Candida* species to host cells is critical for establishing a disease, the components on the *Candida* cell surface involved in this process have been a focus of intensive investigation.

ALS/EPA Adhesins (also see chapter 16)

C. albicans ALS1 was originally identified as a hypha-induced gene whose product shares sequence similarity to the *S. cerevisiae* agglutinin AGα1, which promotes the cell-cell adhesion of mating partners (93), though structurally and functionally, they are more similar to the FLO family of flocculins in yeast. Additional *ALS* genes were soon found, and this protein family is currently appreciated to consist of eight cell surface GPI-anchored proteins that mediate binding to the host cells (82, 90, 203, 233). Each ALS protein has three domains. The substrate binding domain at the N terminus consists of a 127-residue conserved immunoglobulin-like domain (92, 132, 175, 203). The central domain

contains a variable number of 36-residue tandem repeat sequences, which are predicted to fold into a compact protein core surrounded by extensive N- and O-linked glycosylation. The tandem-repeat domain can mediate ALS interactions with both proteins, including fibronectin, and carbohydrates (72, 175). Several ALS proteins (ALS1, ALS3, and ALS5) have a heptapeptide sequence predicted to have amyloid-forming potential, and the ALS5 sequence does form amyloid-like aggregates (160, 175). The Ser/Thr-rich C-terminal domain contains a GPI anchor sequence to cross-link the ALS proteins to the cell wall (92).

The role of ALS in adherence has been widely studied (see Table 3). Heterologous expression studies have determined that the different ALS proteins have distinct yet overlapping profiles of adherence to host cells. Als1p mediates the adherence to FaDu epithelial cells, human umbilical vein endothelial cells (HUVEC), and vascular endothelial cell monolayers but adheres poorly to human buccal epithelial cells (78, 233). *als1*Δ/Δ mutants were modestly attenuated in the mouse systemic model and showed somewhat reduced damage to reconstituted human epithelial (RHE) layers, perhaps due to altered germ tube formation (233). The highly homologous Als2 and Als4 both seem to be involved in adherence to vascular endothelial cells (233, 236). Deletion of *ALS2* allele also resulted in decreased adhesion and destruction of RHE layers (233, 236). Als5 has been reported to mediate the binding to fibronectin, type IV collagen, and laminin (82). However, a more recent study has demonstrated that deletion of *ALS5*, *ALS6*, or *ALS7* resulted in increased adherence to epithelial cells and endothelial cells, suggesting antiadhesive roles for the corresponding ALS family members (234). It is possible that the lack of these proteins induces compensatory mechanisms (234), though this is not well understood. Deletion of *ALS9* did not have any effect on *C. albicans* adherence or damage in the RHE model, suggesting that it is not necessary for adherence to epithelial cells (235).

Als3p is perhaps the most interesting of these proteins. Like Als1p, it is hypha specific and is involved in adhesion to epithelial and endothelial cells (41, 159). Blocking ALS3 function via genetic deletion or ALS3-specific antibodies decreased binding to endothelial cells, buccal epithelial cells, and FaDu monolayers (118, 159, 236), and *als3*Δ/Δ mutants caused less damage to epithelial cells in an RHE model (233). Als3p induces clathrin-dependent internalization of *C. albicans* via specific interations with N- or E-cadherin (141, 168). Latex beads coated with recombinant N-terminal region of Als3p were internalized as efficiently as whole cells, indicating sufficiency (168). It also likely plays a role in adherence between *C. albicans* cells, as *als3*Δ/Δ mutants show aberrant biofilm formation (157). Als3p also binds ferritin and plays an important role in the acquisition of iron in the host (5). The gene is upregulated in clinical vaginal fluid specimens and in a vaginal candidiasis model (36), but a systemic virulence phenotype has not been reported.

The multiplicity of the *ALS* genes makes the phenotypic analysis of these strains particularly difficult. This could be due both to the functional redundancy of other ALS and non-ALS adhesins and to the compensatory upregulation of other adhesion genes (203, 236). For example, strains lacking *ALS2* showed about a 40% reduction in adherence, and a 30% reduction in adherence was observed in a strain lacking *ALS4*. Yet, there was a 2.8-fold-increased expression of *ALS2* in the *als4*Δ mutant strain and a 3.2-fold induction of *ALS4* in the *als2*Δ mutant (236). It seems certain that the *ALS* genes as a family are essential during systemic infections and almost as certain that this will be difficult to definitively demonstrate.

Orthologs of *ALS* genes are present in all the CUG *Candida* species (25, 56, 102), but their functions have not been studied. In *C. glabrata*, an analogous protein family, named EPA for epithelial adhesin, has been investigated in some detail. EPA1 was first identified in a screen for transposon

TABLE 3 Major adhesins in *C. albicans* and *C. glabrata*[a]

Gene mutated	Epithelial cells: Buccal	Epithelial cells: FaDu monolayers	Endothelial cells, HUVEC monolayer	Fibronectin	Laminin	Reference(s)
ALS/EPA						
ALS1	NC	NC/Down	Down	NC	NC	3, 76, 91, 233
ALS2	NC	NT	Down	NC	NC	236
ALS3	Down	Down	Down	NC	NT	233
ALS4	NC	NT	Down	NT	NT	236
ALS5	Up	NT	Up	NT	NT	234
ALS6	Up	NT	Up	NT	NT	234
ALS7	Up	NT	Up	NT	NT	234
ALS9	NC	NT	Down	NC	NT	235
EPA1	Down[b]		NT	NT	NT	31, 42, 52
EPA1,6,7	Down[b]		NT	NT	NT	52
Other adhesins						
IFF4	Up[c]	NT	NC[c]	NT	NT	77
IFF11	Down	NT	NT	NT	NT	14
HWP1	Down	NT	NT	NT	NT	208

[a]Effect on adherence of mutation of the indicated gene(s). Down, lower adherence than with the wild type; up, higher adherence than with the wild type; NC, no change in adhesion; NT, not tested.

[b]Tested in HEp-2, Lec2, T24, and A498 epithelial cell lines.

[c]Effects shown are of overexpression of IFF4. Deletion does not affect adherence.

insertions that blocked adhesion to epithelial cells (42), and it later emerged that the *EPA* family is even larger than the *ALS* family, with 17 to 23 genes, depending on the strain (106). EPA proteins are GPI-anchored lectins with a similar overall structure to ALS and FLO, though they share little primary sequence homology. Most of these genes are encoded in subtelomeric clusters and are transcriptionally silenced; EPA1 is an exception, explaining why the single *epa1*Δ mutant had such a pronounced in vitro phenotype (48). The silencing depends on many *trans*-acting factors shown to be involved in subtelomeric silencing in *S. cerevisiae*, including SIR2, SIR3, SIR4, RAP1, and RIF1 (31, 101, 102). Decreased expression of those factors results in hyperadhesion in *C. glabrata* and enhanced colonization of kidneys in a murine model of disseminated candidiasis (31). *EPA1*, *EPA6*, and *EPA7* were found to be highly expressed in *C. glabrata* cells recovered from the bladders of mice, apparently in response to nicotinic acid starvation in this niche (52). Deletion of these three genes reduced colonization of the bladder about 10-fold, confirming a role for these proteins in this niche while also suggesting other mechanisms that mediate colonization in the absence of EPA1, -6, and -7 (52).

IFF Family

HYR1 is the founding member of the awkwardly named IFF family, which derives its name from a systematic analysis of protein families (individual protein family F), which has stuck for lack of a deeper biological understanding of their function. This is the largest family of putative cell wall proteins in *C. albicans*, with 12 members (HYR1 and IFF1 to -11), though much less is known about it than the ALS family (see Table 3). Deletion of HYR1 (hyphal regulated) has no apparent phenotype (11), and not all *IFF* genes are regulated by morphology. Two other IFF proteins have been studied further, IFF4 and IFF11. Overexpression of Iff4 was shown to increase adherence to oral epithelial cells (77) and is required for virulence in disseminated candidiasis and in a mouse model of vaginal candidiasis (77, 107). The *iff4*Δ/Δ mutant is more susceptible to neutrophil killing and has reduced virulence in a systematic model (77). Alone among the IFF/HYR proteins, IFF11 does not have a GPI motif and is secreted (14). A *C. albicans* strain lacking IFF11 was hypersensitive to cell wall-damaging agents, suggesting a role in cell wall organization. In addition, this strain showed markedly reduced virulence in a mouse model of disseminated candidiasis, suggesting that Iff11 is necessary for establishment of infection (14).

HWP1

HWP1 is a GPI-anchored adhesin expressed as befits its name. The proline-rich N-terminal region of HWP1 is covalently linked by epithelial cell-associated transglutaminases to other proteins on the host cell surface (172, 208), possibly as a host cell mimic. *C. albicans* strains lacking *HWP1* failed to adhere to human buccal epithelial cells and had a reduced virulence during both mucosal and systemic infections (208, 213). HWP1-dependent attachment occurred in mature buccal epithelial cells displaying SPR3 and keratin 13 on their surface, indicating a specificity of interaction (172). Adhesion mediated by HWP1 also contributes to biofilm formation, both in vitro and in an in vivo catheter model, and to efficient mating, which requires cell-cell adhesion as well (60, 156). *HWP1* is also well known for its unfortunate role in illustrating the magnitude of the "*URA3* position effect," in which variable expression of the *URA3* marker, previously used routinely for gene disruptions, based on chromosomal position can affect virulence in a manner that complicates analysis of the target gene (202, 208, 214). Reanalysis of the role of *HWP1* in systemic virulence using strains in which the *URA3* position effect was carefully controlled confirmed that the mutant was attenuated, though not as severely as first reported (208, 214).

Invasion and Damage

Invasion and endocytosis are terms primarily used to describe the internalization of *Candida* into normally nonphagocytic cells, mostly endothelial and epithelial cells for this discussion (reviewed in references 66 and 238). There are four main mechanisms by which *Candida* can penetrate endothelial or epithelial layers: endocytosis into the host cell, direct penetration of host cells, passage between cells through tight junctions, and hitchhiking inside of migrating leukocytes. All are probably relevant in vivo. These mechanisms are illustrated in Fig. 2 in relation to endothelial cells and in a recent review for epithelial cells (238).

Normally nonphagocytic cells such as endothelial and epithelial cells endocytose *C. albicans*. Once the organism is internalized, hyphal growth (of *C. albicans*) disrupts the cells, allowing the pathogen access to the abluminal compartment. This disruption may also allow neighboring *C. albicans* cells (and, on epithelial surfaces, other microbes) to penetrate into host tissue as part of a "jailbreak." Endocytosis is triggered by a specific calcium-dependent interaction between ALS3 and host cadherins, either N-cadherin on endothelial cells or E-cadherin on epithelial cells (141, 167, 168). Endocytosis begins with the hyphal tips, where ALS3 localizes (see Fig. 2), and can be blocked by anti-ALS3 antibodies (168). The endocytic process is driven by the host cell, since killed hyphae were internalized as efficiently as live ones, and is dependent on clathrin, dynamin, and cortactin, since small interfering RNA depletion of these proteins significantly reduced *C. albicans* internalization, as does inhibition of actin polymerization after treatment with cytochalasin D (141, 164). Importantly, these treatments do not completely block endocytosis, suggesting that additional, as-yet-unknown, mechanisms also mediate endocytosis. Despite these in vitro observations, endocytosis and pseudopod formation have not been directly observed in vivo.

C. albicans can also directly penetrate epithelial and endothelial layers either by disrupting cell-cell junctions and extracellular matrices and passing between cells or by entering cells independent of endocytosis. The SAPs are likely required for this, as pepstatin A inhibits lesions formed by *C. albicans* in a mouse skin model (176). Results obtained using individual *sap*Δ mutants have been confusing, with some papers describing defects and others not (122, 196). Pepstatin also reduces tissue damage caused by *C. dubliniensis* in a keratitis model, with two of the eight SAPs (SAP3 and SAP4) apparently the primary targets (126). The PLD1 phospholipase is also required for maximal adhesion and invasion of epithelial cells (121). It is logical that other extracellular hydrolases also mediate tissue damage, but there is limited evidence for this.

Invasion of epithelial or endothelial layers can cause a breakdown of integrity, as measured by increased permeability to large dyes (such as dextran), chromium release from cells, and decreased electrical resistance across the layer. This can occur following endocytosis or degradation of tight junctions, so the result of both mechanisms is the leakage

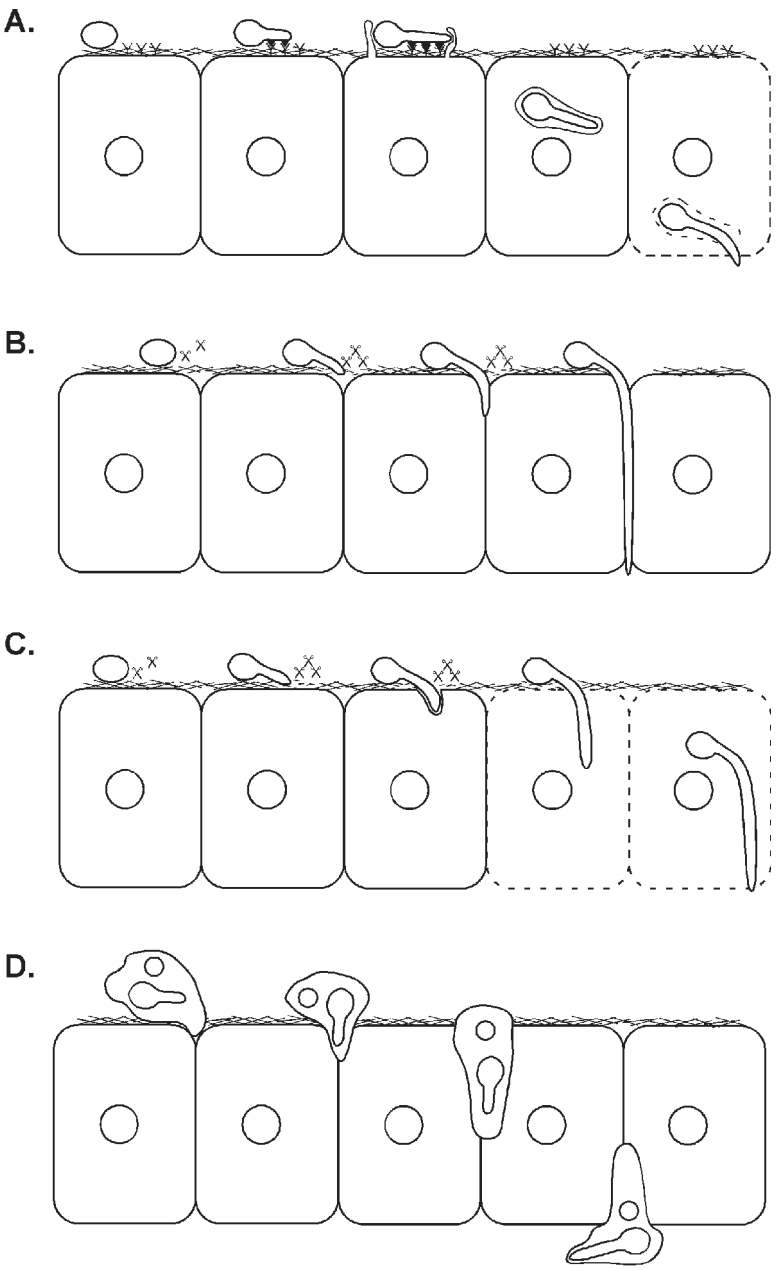


FIGURE 2 Mechanisms by which *C. albicans* can cross endothelial layers. (A) Endocytosis can be triggered by specific interactions between ALS3 (triangles) and N-cadherin ("Y"), which leads to pseudopod formation and internalization. Hyphal morphogenesis inside the endothelial cell can lead to rupture and escape. (B) Passage between cells by degrading extracellular matrix and tight junctions. Scissors represent secreted proteases. (C) Passage through the host cell. Scissors represent secreted proteases and/or lipases. (D) Transit within phagocytes. A phagocytosed *C. albicans* cell crosses the endothelial layer within a migrating phagocyte. Many of these mechanisms are also applicable to *C. albicans* interactions with epithelial cells; see reference 238 for greater detail on host epithelial interactions. 10.1128/9781555817176.ch17f2

of luminal contents, potentially including other microbes, across this layer. The mechanisms by which *C. albicans* causes damage are not well understood, but hyphal growth is thought to be a main cause. A few genes not directly associated with morphogenesis have been shown to reduce damage as well, including *VPS51* (vacuolar protein sorting) and a novel gene, *UEC1* (163). Damage is not an inevitable result, however, as *C. albicans* penetrates the blood-brain barrier without obviously impairing integrity (104).

Finally, *Candida* cells could penetrate the endothelium, and potentially the epithelium, by hitching a ride inside of migrating phagocytes. These cells pass across layers and into

tissues without damaging the endothelial cells or integrity of this cell layer. *C. albicans* has been seen inside neutrophils in peripheral blood in patients with candidiasis (143), but what role this might play in dissemination in vivo is not clear. There is more evidence, however, that *Cryptococcus neoformans* uses this mechanism, perhaps as a way to transit the blood-brain barrier.

CONCLUSIONS AND PERSPECTIVES

Candida species, especially *C. albicans*, have an extended evolutionary history in contact with the mammalian host, particularly with a few cell types. These interactions have been extensive enough that this pathogen has developed specific responses to each cell type, with only limited overlap between them: *C. albicans* cells upregulate oxidative stress responses when confronted by macrophages or neutrophils, but the remainder of the complex transcriptional programs are unique. At least some of this is simply a result of recognizing environmental conditions in each niche, as the divergent *C. glabrata* shares with *C. albicans* a very similar response to macrophages. Pathogens like *Candida* species have to overcome a variety of extracellular stresses that are rarely encountered by nonpathogens. As a result, these organisms have moved many aspects of stress responses to the outside of the cell; the roles of the SODs, catalases, and proteases secreted into the medium or onto the cell wall are a case in point. Similarly, adhesion to substrates is important in organisms in many environments, but at least two of the many adhesins in *C. albicans* have very precise roles in the host-pathogen interaction, ALS3 as an invasin that promotes endocytosis and translocation across endothelial and epithelial layers (168) and HWP1 as a transglutaminase substrate enabling tight, covalent interactions with host cells (209). A conceptually similar story emerges from *C. glabrata*, where the EPAs are differentially regulated by body site and contribute to virulence in a tissue-specific manner (42, 48, 52). Available data indicate that these adaptations are critical for virulence, though much work remains to be done to clarify the roles of most of these proteins.

As this chapter has shown, many of these responses were originally discovered as a result of genetics, biochemistry, and hard work. The importance of the pioneering work done by many people on secreted proteases, the ALS family, morphogenesis, and host cell interactions is often underappreciated: knockout phenotypes were often unimpressive, genetic methodology was still under development, and subtle changes in experimental design, cell types, and strains sometimes had profound effects on expression of some of these factors. Today, with the benefit of genomic-scale technologies and complete genome sequence of multiple isolates and species, we can see that these early results were the first hints of the remarkable complexity of the *Candida*-host interaction. We are just beginning to clearly understand the roles of a few of these important mediators, such as ALS3—and generally only in a single species, *C. albicans* (and, in cases, *C. glabrata*). Complete clarity still needs to be established.

REFERENCES

1. **Abe, S., R. Tsunashima, R. Iijima, T. Yamada, N. Maruyama, T. Hisajima, Y. Abe, H. Oshima, and M. Yamazaki.** 2009. Suppression of anti-*Candida* activity of macrophages by a quorum-sensing molecule, farnesol, through induction of oxidative stress. *Microbiol. Immunol.* **53:**323–330.
2. **Alarco, A. M., and M. Raymond.** 1999. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J. Bacteriol.* **181:**700–708.
3. **Alberti-Segui, C., A. J. Morales, H. Xing, M. M. Kessler, D. A. Willins, K. G. Weinstock, G. Cottarel, K. Fechtel, and B. Rogers.** 2004. Identification of potential cell-surface proteins in *Candida albicans* and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence. *Yeast* **21:**285–302.
4. **Albrecht, A., A. Felk, I. Pichova, J. R. Naglik, M. Schaller, P. de Groot, D. Maccallum, F. C. Odds, W. Schafer, F. Klis, M. Monod, and B. Hube.** 2006. Glycosylphosphatidylinositol-anchored proteases of *Candida albicans* target proteins necessary for both cellular processes and host-pathogen interactions. *J. Biol. Chem.* **281:**688–694.
5. **Almeida, R. S., S. Brunke, A. Albrecht, S. Thewes, M. Laue, J. E. Edwards, S. G. Filler, and B. Hube.** 2008. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog.* **4:**e1000217.
6. **Alonso-Monge, R., F. Navarro-Garcia, G. Molero, R. Diez-Orejas, M. Gustin, J. Pla, M. Sanchez, and C. Nombela.** 1999. Role of the mitogen-activated protein kinase Hog1p in morphogenesis and virulence of *Candida albicans*. *J. Bacteriol.* **181:**3058–3068.
7. **Alonso-Monge, R., F. Navarro-Garcia, E. Roman, A. I. Negredo, B. Eisman, C. Nombela, and J. Pla.** 2003. The Hog1 mitogen-activated protein kinase is essential in the oxidative stress response and chlamydospore formation in *Candida albicans*. *Eukaryot. Cell* **2:**351–361.
8. **Alvarez-Peral, F. J., O. Zaragoza, Y. Pedreno, and J. C. Arguelles.** 2002. Protective role of trehalose during severe oxidative stress caused by hydrogen peroxide and the adaptive oxidative stress response in *Candida albicans*. *Microbiology* **148:**2599–2606.
9. **Andes, D., A. Lepak, A. Pitula, K. Marchillo, and J. Clark.** 2005. A simple approach for estimating gene expression in *Candida albicans* directly from a systemic infection site. *J. Infect. Dis.* **192:**893–900.
10. **Arguelles, J. C.** 2000. Physiological roles of trehalose in bacteria and yeasts: a comparative analysis. *Arch. Microbiol.* **174:**217–224.
11. **Bailey, D. A., P. J. Feldmann, M. Bovey, N. A. Gow, and A. J. Brown.** 1996. The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J. Bacteriol.* **178:**5353–5360.
12. **Barelle, C. J., C. L. Priest, D. M. Maccallum, N. A. Gow, F. C. Odds, and A. J. Brown.** 2006. Niche-specific regulation of central metabolic pathways in a fungal pathogen. *Cell. Microbiol.* **8:**961–971.
13. **Barker, K. S., H. Park, Q. T. Phan, L. Xu, R. Homayouni, P. D. Rogers, and S. G. Filler.** 2008. Transcriptome profile of the vascular endothelial cell response to *Candida albicans*. *J. Infect. Dis.* **198:**193–202.
14. **Bates, S., J. M. de la Rosa, D. M. MacCallum, A. J. Brown, N. A. Gow, and F. C. Odds.** 2007. *Candida albicans* Iff11, a secreted protein required for cell wall structure and virulence. *Infect. Immun.* **75:**2922–2928.
15. **Bensen, E. S., S. J. Martin, M. Li, J. Berman, and D. A. Davis.** 2004. Transcriptional profiling in *Candida albicans* reveals new adaptive responses to extracellular pH and functions for Rim101p. *Mol. Microbiol.* **54:**1335–1351.
16. **Bernhardt, J., D. Herman, M. Sheridan, and R. Calderone.** 2001. Adherence and invasion studies of *Candida albicans* strains, using in vitro models of esophageal candidiasis. *J. Infect. Dis.* **184:**1170–1175.

17. **Bethea, E. K., B. J. Carver, A. E. Montedonico, and T. B. Reynolds.** 2010. The inositol regulon controls viability in *Candida glabrata*. *Microbiology* **156:**452–462.
18. **Borg, M., and R. Ruchel.** 1990. Demonstration of fungal proteinase during phagocytosis of *Candida albicans* and *Candida tropicalis*. *J. Med. Vet. Mycol.* **28:**3–14.
19. **Borg-von Zepelin, M., S. Beggah, K. Boggian, D. Sanglard, and M. Monod.** 1998. The expression of the secreted aspartyl proteinases Sap4 to Sap6 from *Candida albicans* in murine macrophages. *Mol. Microbiol.* **28:**543–554.
20. **Brand, A., D. M. MacCallum, A. J. Brown, N. A. Gow, and F. C. Odds.** 2004. Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintegration of *URA3* at the *RPS10* locus. *Eukaryot. Cell* **3:** 900–909.
21. **Brasch, J., J. M. Schroder, and E. Christophers.** 1992. *Candida albicans* grown in glucose-free media contains serum-independent chemotactic activity. *Acta Derm. Venereol.* **72:**1–3.
22. **Braun, B. R., D. Kadosh, and A. D. Johnson.** 2001. *NRG1*, a repressor of filamentous growth in *C. albicans*, is down-regulated during filament induction. *EMBO J.* **20:** 4753–4761.
23. **Brown, A. J.** 2002. Expression of growth form-specific factors during morphogenesis in *Candida albicans*, p. 87–94. *In* R. Calderone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
24. **Brown, A. J.** 2002. Morphogenetic signaling pathways in *Candida albicans*, p. 95–106. *In* R. Calderone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
25. **Butler, G., M. D. Rasmussen, M. F. Lin, M. A. Santos, S. Sakthikumar, C. A. Munro, E. Rheinbay, M. Grabherr, A. Forche, J. L. Reedy, I. Agrafioti, M. B. Arnaud, S. Bates, A. J. Brown, S. Brunke, M. C. Costanzo, D. A. Fitzpatrick, P. W. de Groot, D. Harris, L. L. Hoyer, B. Hube, F. M. Klis, C. Kodira, N. Lennard, M. E. Logue, R. Martin, A. M. Neiman, E. Nikolaou, M. A. Quail, J. Quinn, M. C. Santos, F. F. Schmitzberger, G. Sherlock, P. Shah, K. A. Silverstein, M. S. Skrzypek, D. Soll, R. Staggs, I. Stansfield, M. P. Stumpf, P. E. Sudbery, T. Srikantha, Q. Zeng, J. Berman, M. Berriman, J. Heitman, N. A. Gow, M. C. Lorenz, B. W. Birren, M. Kellis, and C. A. Cuomo.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. *Nature* **459:** 657–662.
26. **Calcagno, A. M., E. Bignell, P. Warn, M. D. Jones, D. W. Denning, F. A. Muhlschlegel, T. R. Rogers, and K. Haynes.** 2003. *Candida glabrata* STE12 is required for wild-type levels of virulence and nitrogen starvation induced filamentation. *Mol. Microbiol.* **50:**1309–1318.
27. **Cao, Y., Y. Wang, B. Dai, B. Wang, H. Zhang, Z. Zhu, Y. Xu, Y. Jiang, and G. Zhang.** 2008. Trehalose is an important mediator of Cap1p oxidative stress response in *Candida albicans*. *Biol. Pharm. Bull.* **31:**421–425.
28. **Carman, A. J., S. Vylkova, and M. C. Lorenz.** 2008. Role of acetyl coenzyme a synthesis and breakdown in alternative carbon source utilization in *Candida albicans*. *Eukaryot. Cell* **7:**1733–1741.
29. **Caro, L. H., H. Tettelin, J. H. Vossen, A. F. Ram, H. van den Ende, and F. M. Klis.** 1997. In silicio identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of *Saccharomyces cerevisiae*. *Yeast* **13:**1477–1489.
30. **Casanova, M., J. L. Lopez-Ribot, J. P. Martinez, and R. Sentandreu.** 1992. Characterization of cell wall proteins from yeast and mycelial cells of *Candida albicans* by labelling with biotin: comparison with other techniques. *Infect. Immun.* **60:**4898–4906.
31. **Castano, I., S. J. Pan, M. Zupancic, C. Hennequin, B. Dujon, and B. P. Cormack.** 2005. Telomere length control and transcriptional regulation of subtelomeric adhesins in *Candida glabrata*. *Mol. Microbiol.* **55:**1246–1258.
32. **Chauhan, N., D. Inglis, E. Roman, J. Pla, D. Li, J. A. Calera, and R. Calderone.** 2003. *Candida albicans* response regulator gene *SSK1* regulates a subset of genes whose functions are associated with cell wall biosynthesis and adaptation to oxidative stress. *Eukaryot. Cell* **2:**1018–1024.
33. **Chauhan, N., J. P. Latge, and R. Calderone.** 2006. Signalling and oxidant adaptation in *Candida albicans* and *Aspergillus fumigatus*. *Nat. Rev.* **4:**435–444.
34. **Chaves, G. M., S. Bates, D. M. Maccallum, and F. C. Odds.** 2007. *Candida albicans GRX2*, encoding a putative glutaredoxin, is required for virulence in a murine model. *Genet. Mol. Res.* **6:**1051–1063.
35. **Chen, Y. L., S. Kauffman, and T. B. Reynolds.** 2008. Candida albicans uses multiple mechanisms to acquire the essential metabolite inositol during infection. *Infect. Immun.* **76:**2793–2801.
36. **Cheng, G., K. Wozniak, M. A. Wallig, P. L. Fidel, Jr., S. R. Trupin, and L. L. Hoyer.** 2005. Comparison between *Candida albicans* agglutinin-like sequence gene expression patterns in human clinical specimens and models of vaginal candidiasis. *Infect. Immun.* **73:**1656–1663.
37. **Cheng, G., K. M. Yeater, and L. L. Hoyer.** 2006. Cellular and molecular biology of *Candida albicans* estrogen response. *Eukaryot. Cell* **5:**180–191.
38. **Chinen, T., M. H. Qureshi, Y. Koguchi, and K. Kawakami.** 1999. *Candida albicans* suppresses nitric oxide (NO) production by interferon-gamma (IFN-gamma) and lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. *Clin. Exp. Immunol.* **115:**491–497.
39. **Chiranand, W., I. McLeod, H. Zhou, J. J. Lynn, L. A. Vega, H. Myers, J. R. Yates III, M. C. Lorenz, and M. C. Gustin.** 2008. CTA4 transcription factor mediates induction of nitrosative stress response in *Candida albicans*. *Eukaryot. Cell* **7:**268–278.
40. **Cole, M. F., W. H. Bowen, X. J. Zhao, and R. L. Cihlar.** 1995. Avirulence of *Candida albicans* auxotrophic mutants in a rat model of oropharyngeal candidiasis. *FEMS Microbiol. Lett.* **126:**177–180.
41. **Coleman, D. A., S. H. Oh, X. Zhao, H. Zhao, J. T. Hutchins, J. H. Vernachio, J. M. Patti, and L. L. Hoyer.** 2009. Monoclonal antibodies specific for *Candida albicans* Als3 that immunolabel fungal cells in vitro and in vivo and block adhesion to host surfaces. *J. Microbiol. Methods* **78**:71–78.
42. **Cormack, B. P., N. Ghori, and S. Falkow.** 1999. An adhesin of the yeast pathogen *Candida glabrata* mediating adherence to human epithelial cells. *Science* **285:**578–582.
43. **Crowe, J. D., I. K. Sievwright, G. C. Auld, N. R. Moore, N. A. Gow, and N. A. Booth.** 2003. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. *Mol. Microbiol.* **47:**1637–1651.
44. **Cuellar-Cruz, M., M. Briones-Martin-del-Campo, I. Canas-Villamar, J. Montalvo-Arredondo, L. Riego-Ruiz, I. Castano, and A. De Las Penas.** 2008. High resistance to oxidative stress in the fungal pathogen *Candida glabrata* is mediated by a single catalase, Cta1p, and is controlled by the transcription factors Yap1p, Skn7p, Msn2p, and Msn4p. *Eukaryot. Cell* **7:**814–825.
45. **Cutler, J. E.** 1977. Chemotactic factor produced by *Candida albicans*. *Infect. Immun.* **18:**568–573.
46. **de Groot, P. W., A. D. de Boer, J. Cunningham, H. L. Dekker, L. de Jong, K. J. Hellingwerf, C. de Koster, and F. M. Klis.** 2004. Proteomic analysis of *Candida albicans* cell walls reveals covalently bound carbohydrate-active enzymes and adhesins. *Eukaryot. Cell* **3:**955–965.

47. **De Groot, P. W., K. J. Hellingwerf, and F. M. Klis.** 2003. Genome-wide identification of fungal GPI proteins. *Yeast* **20:**781–796.
48. **De Las Penas, A., S. J. Pan, I. Castaäno, J. Alder, R. Cregg, and B. P. Cormack.** 2003. Virulence-related surface glycoproteins in the yeast pathogen *Candida glabrata* are encoded in subtelomeric clusters and subject to RAP1- and SIR-dependent transcriptional silencing. *Genes Dev.* **17:**2245–2258.
49. **Dieterich, C., M. Schandar, M. Noll, F. J. Johannes, H. Brunner, T. Graeve, and S. Rupp.** 2002. In vitro reconstructed human epithelia reveal contributions of *Candida albicans* EFG1 and CPH1 to adhesion and invasion. *Microbiology* **148:**497–506.
50. **do Carmo-Sousa, L.** 1969. Distribution of yeasts in nature, p. 79–105. *In* A. H. Rose and J. S. Harrison (ed.), *The Yeasts*, vol. 1. Academic Press, London, United Kingdom.
51. **Dolan, J. W., A. C. Bell, B. Hube, M. Schaller, T. F. Warner, and E. Balish.** 2004. *Candida albicans* PLD I activity is required for full virulence. *Med. Mycol.* **42:**439–447.
52. **Domergue, R., I. Castano, A. De Las Penas, M. Zupancic, V. Lockatell, J. R. Hebel, D. Johnson, and B. P. Cormack.** 2005. Nicotinic acid limitation regulates silencing of *Candida* adhesins during UTI. *Science* **308:**866–870.
53. **Donovan, M., J. J. Schumuke, W. A. Fonzi, S. L. Bonar, K. Gheesling-Mullis, G. S. Jacob, V. J. Davisson, and S. B. Dotson.** 2001. Virulence of a phosphoribosylaminoimidazole carboxylase-deficient *Candida albicans* strain in an immunosuppressed murine model of systemic candidiasis. *Infect. Immun.* **69:**2542–2548.
54. **Draculic, T., I. W. Dawes, and C. M. Grant.** 2000. A single glutaredoxin or thioredoxin gene is essential for viability in the yeast *Saccharomyces cerevisiae*. *Mol. Microbiol.* **36:**1167–1174.
55. **Du, C., R. Calderone, J. Richert, and D. Li.** 2005. Deletion of the *SSK1* response regulator gene in *Candida albicans* contributes to enhanced killing by human polymorphonuclear neutrophils. *Infect. Immun.* **73:**865–871.
56. **Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. De Montigny, C. Marck, C. Neuveglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J. M. Beckerich, E. Beyne, C. Bleykasten, A. Boisrame, J. Boyer, L. Cattolico, F. Confanioleri, A. De Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J. M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G. F. Richard, M. L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wesolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J. L. Souciet.** 2004. Genome evolution in yeasts. *Nature* **430:**35–44.
57. **Edens, H. A., C. A. Parkos, T. W. Liang, A. J. Jesaitis, J. E. Cutler, and H. M. Miettinen.** 1999. Non-serum-dependent chemotactic factors produced by *Candida albicans* stimulate chemotaxis by binding to the formyl peptide receptor on neutrophils and to an unknown receptor on macrophages. *Infect. Immun.* **67:**1063–1071.
58. **Eisenhaber, B., G. Schneider, M. Wildpaner, and F. Eisenhaber.** 2004. A sensitive predictor for potential GPI lipid modification sites in fungal protein sequences and its application to genome-wide studies for *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *J. Mol. Biol.* **337:**243–253.
59. **Elson, S. L., S. M. Noble, N. V. Solis, S. G. Filler, and A. D. Johnson.** 2009. An RNA transport system in *Candida albicans* regulates hyphal morphology and invasive growth. *PLoS Genet.* **5:**e1000664.
60. **Ene, I. V., and R. J. Bennett.** 2009. Hwp1 and related adhesins contribute to both mating and biofilm formation in *Candida albicans*. *Eukaryot. Cell* **8:**1909–1913.
61. **Enjalbert, B., D. M. MacCallum, F. C. Odds, and A. J. Brown.** 2007. Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. *Infect. Immun.* **75:**2143–2151.
62. **Enjalbert, B., A. Nantel, and M. Whiteway.** 2003. Stress-induced gene expression in *Candida albicans*: absence of a general stress response. *Mol. Biol. Cell* **14:**1460–1467.
63. **Enjalbert, B., D. A. Smith, M. J. Cornell, I. Alam, S. Nicholls, A. J. Brown, and J. Quinn.** 2006. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. *Mol. Biol. Cell* **17:**1018–1032.
64. **Fernandez-Arenas, E., C. K. Bleck, C. Nombela, C. Gil, G. Griffiths, and R. Diez-Orejas.** 2009. *Candida albicans* actively modulates intracellular membrane trafficking in mouse macrophage phagosomes. *Cell. Microbiol.* **11:**560–589.
65. **Fernandez-Arenas, E., V. Cabezon, C. Bermejo, J. Arroyo, C. Nombela, R. Diez-Orejas, and C. Gil.** 2007. Integrated proteomics and genomics strategies bring new insight into *Candida albicans* response upon macrophage interaction. *Mol. Cell. Proteomics* **6:**460–478.
66. **Filler, S. G., and D. C. Sheppard.** 2006. Fungal invasion of normally non-phagocytic host cells. *PLoS Pathog.* **2:**e129.
67. **Filler, S. G., J. N. Swerdloff, C. Hobbs, and P. M. Luckett.** 1995. Penetration and damage of endothelial cells by *Candida albicans*. *Infect. Immun.* **63:**976–983.
68. **Forsyth, C. B., and H. L. Mathews.** 1996. Lymphocytes utilize CD11b/CD18 for adhesion to *Candida albicans*. *Cell. Immunol.* **170:**91–100.
69. **Forsyth, C. B., E. F. Plow, and L. Zhang.** 1998. Interaction of the fungal pathogen *Candida albicans* with integrin CD11b/CD18: recognition by the I domain is modulated by the lectin-like domain and the CD18 subunit. *J. Immunol.* **161:**6198–6205.
70. **Fradin, C., P. De Groot, D. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube.** 2005. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol. Microbiol.* **56:**397–415.
71. **Fradin, C., M. Kretschmar, T. Nichterlein, C. Gaillardin, C. d'Enfert, and B. Hube.** 2003. Stage-specific gene expression of *Candida albicans* in human blood. *Mol. Microbiol.* **47:**1523–1543.
72. **Frank, A. T., C. Ramsook, H. N. Otoo, C. Tan, G. Soybelman, J. M. Rauceo, N. K. Gaur, S. A. Klotz, and P. N. Lipke.** 2010. Structure and function of glycosylated tandem repeats from *Candida albicans* Als adhesins. *Eukaryot. Cell* **9:**405–414.
73. **Frealle, E., C. Noel, E. Viscogliosi, D. Camus, E. Dei-Cas, and L. Delhaes.** 2005. Manganese superoxide dismutase in pathogenic fungi: an issue with pathophysiological and phylogenetic involvements. *FEMS Immunol. Med. Microbiol.* **45:**411–422.
74. **Frohner, I. E., C. Bourgeois, K. Yatsyk, O. Majer, and K. Kuchler.** 2009. *Candida albicans* cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance. *Mol. Microbiol.* **71:**240–252.
75. **Fu, Y., A. S. Ibrahim, W. Fonzi, X. Zhou, C. F. Ramos, and M. A. Ghannoum.** 1997. Cloning and characteriza-

tion of a gene (LIP1) which encodes a lipase from the pathogenic yeast *Candida albicans*. *Microbiology* **143**(Pt. 2)**:** 331–340.

76. **Fu, Y., A. S. Ibrahim, D. C. Sheppard, Y. C. Chen, S. W. French, J. E. Cutler, S. G. Filler, and J. E. Edwards, Jr.** 2002. *Candida albicans* Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. *Mol. Microbiol.* **44:**61–72.
77. **Fu, Y., G. Luo, B. J. Spellberg, J. E. Edwards, Jr., and A. S. Ibrahim.** 2008. Gene overexpression/suppression analysis of candidate virulence factors of *Candida albicans*. *Eukaryot. Cell* **7:**483–492.
78. **Fu, Y., G. Rieg, W. A. Fonzi, P. H. Belanger, J. E. Edwards, Jr., and S. G. Filler.** 1998. Expression of the *Candida albicans* gene *ALS1* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells. *Infect. Immun.* **66:**1783–1786.
79. **Gacser, A., F. Stehr, C. Kroger, L. Kredics, W. Schafer, and J. D. Nosanchuk.** 2007. Lipase 8 affects the pathogenesis of *Candida albicans*. *Infect. Immun.* **75:**4710–4718.
80. **Gacser, A., D. Trofa, W. Schafer, and J. D. Nosanchuk.** 2007. Targeted gene deletion in *Candida parapsilosis* demonstrates the role of secreted lipase in virulence. *J. Clin. Investig.* **117:**3049–3058.
81. **Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown.** 2000. Genomic expression programs in the response of yeast cells to environmental changes. *Mol. Biol. Cell* **11:**4241–4257.
82. **Gaur, N. K., and S. A. Klotz.** 1997. Expression, cloning, and characterization of a *Candida albicans* gene, *ALA1*, that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins. *Infect. Immun.* **65:**5289–5294.
83. **Geiger, J., D. Wessels, S. R. Lockhart, and D. R. Soll.** 2004. Release of a potent polymorphonuclear leukocyte chemoattractant is regulated by white-opaque switching in *Candida albicans*. *Infect. Immun.* **72:**667–677.
84. **Ghosh, S., D. H. Navarathna, D. D. Roberts, J. T. Cooper, A. L. Atkin, T. M. Petro, and K. W. Nickerson.** 2009. Arginine-induced germ tube formation in *Candida albicans* is essential for escape from murine macrophage line RAW 264.7. *Infect. Immun.* **77:**1596–1605.
85. **Gonzalez, M. I., R. Stucka, M. A. Blazquez, H. Feldmann, and C. Gancedo.** 1992. Molecular cloning of CIF1, a yeast gene necessary for growth on glucose. *Yeast* **8:** 183–192.
86. **Hajjeh, R. A., A. N. Sofair, L. H. Harrison, G. M. Lyon, B. A. Arthington-Skaggs, S. A. Mirza, M. Phelan, J. Morgan, W. Lee-Yang, M. A. Ciblak, L. E. Benjamin, L. T. Sanza, S. Huie, S. F. Yeo, M. E. Brandt, and D. W. Warnock.** 2004. Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J. Clin. Microbiol.* **42:**1519–1527.
87. **Heidenreich, S., B. Otte, D. Lang, and M. Schmidt.** 1996. Infection by *Candida albicans* inhibits apoptosis of human monocytes and monocytic U937 cells. *J. Leukoc. Biol.* **60:**737–743.
88. **Herrero, E., J. Ros, G. Belli, and E. Cabiscol.** 2008. Redox control and oxidative stress in yeast cells. *Biochim. Biophys. Acta* **1780:**1217–1235.
89. **Hornbach, A., A. Heyken, L. Schild, B. Hube, J. Loffler, and O. Kurzai.** 2009. The glycosylphosphatidylinositol-anchored protease Sap9 modulates the interaction of *Candida albicans* with human neutrophils. *Infect. Immun.* **77:**5216–5224.
90. **Hoyer, L. L.** 2001. The ALS gene family of *Candida albicans*. *Trends Microbiol.* **9:**176–180.
91. **Hoyer, L. L., C. B. Green, S. H. Oh, and X. Zhao.** 2008. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family—a sticky pursuit. *Med. Mycol.* **46:**1–15.
92. **Hoyer, L. L., and J. E. Hecht.** 2001. The ALS5 gene of *Candida albicans* and analysis of the Als5p N-terminal domain. *Yeast* **18:**49–60.
93. **Hoyer, L. L., S. Scherer, A. R. Shatzman, and G. P. Livi.** 1995. *Candida albicans* ALS1: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. *Mol. Microbiol.* **15:**39–54.
94. **Hromatka, B. S., S. M. Noble, and A. D. Johnson.** 2005. Transcriptional response of *Candida albicans* to nitric oxide and the role of the *YHB1* gene in nitrosative stress and virulence. *Mol. Biol. Cell* **16:**4814–4826.
95. **Hube, B., D. Sanglard, F. C. Odds, D. Hess, M. Monod, W. Schafer, A. J. Brown, and N. A. Gow.** 1997. Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. *Infect. Immun.* **65:**3529–3538.
96. **Hube, B., F. Stehr, M. Bossenz, A. Mazur, M. Kretschmar, and W. Schafer.** 2000. Secreted lipases of *Candida albicans*: cloning, characterisation and expression analysis of a new gene family with at least ten members. *Arch. Microbiol.* **174:**362–374.
97. **Hwang, C. S., Y. U. Baek, H. S. Yim, and S. O. Kang.** 2003. Protective roles of mitochondrial manganese-containing superoxide dismutase against various stresses in *Candida albicans*. *Yeast* **20:**929–941.
98. **Hwang, C. S., G. Rhie, S. T. Kim, Y. R. Kim, W. K. Huh, Y. U. Baek, and S. O. Kang.** 1999. Copper- and zinc-containing superoxide dismutase and its gene from *Candida albicans*. *Biochim. Biophys. Acta* **1427:**245–255.
99. **Hwang, C. S., G. E. Rhie, J. H. Oh, W. K. Huh, H. S. Yim, and S. O. Kang.** 2002. Copper- and zinc-containing superoxide dismutase (Cu/ZnSOD) is required for the protection of *Candida albicans* against oxidative stresses and the expression of its full virulence. *Microbiology* **148:** 3705–3713.
100. **Ibata-Ombetta, S., T. Idziorek, P. A. Trinel, D. Poulain, and T. Jouault.** 2003. *Candida albicans* phospholipomannan promotes survival of phagocytosed yeasts through modulation of bad phosphorylation and macrophage apoptosis. *J. Biol. Chem.* **278:**13086–13093.
101. **Iraqui, I., S. Garcia-Sanchez, S. Aubert, F. Dromer, J. M. Ghigo, C. d'Enfert, and G. Janbon.** 2005. The Yak1p kinase controls expression of adhesins and biofilm formation in *Candida glabrata* in a Sir4p-dependent pathway. *Mol. Microbiol.* **55:**1259–1271.
102. **Jackson, A. P., J. A. Gamble, T. Yeomans, G. P. Moran, D. Saunders, D. Harris, M. Aslett, J. F. Barrell, G. Butler, F. Citiulo, D. C. Coleman, P. W. de Groot, T. J. Goodwin, M. A. Quail, J. McQuillan, C. A. Munro, A. Pain, R. T. Poulter, M. A. Rajandream, H. Renauld, M. J. Spiering, A. Tivey, N. A. Gow, B. Barrell, D. J. Sullivan, and M. Berriman.** 2009. Comparative genomics of the fungal pathogens *Candida dubliniensis* and *Candida albicans*. *Genome Res.* **19:**2231–2244.
103. **Jacobsen, I. D., S. Brunke, K. Seider, T. Schwarzmuller, A. Firon, C. d'Enfert, K. Kuchler, and B. Hube.** 2010. *Candida glabrata* persistence in mice does not depend on host immunosuppression and is unaffected by fungal amino acid auxotrophy. *Infect. Immun.* **78:**1066–1077.
104. **Jong, A. Y., M. F. Stins, S. H. Huang, S. H. Chen, and K. S. Kim.** 2001. Traversal of *Candida albicans* across human blood-brain barrier in vitro. *Infect. Immun.* **69:** 4536–4544.
105. **Kaposzta, R., L. Marodi, M. Hollinshead, S. Gordon, and R. P. da Silva.** 1999. Rapid recruitment of late

endosomes and lysosomes in mouse macrophages ingesting *Candida albicans*. *J. Cell Sci.* **112**(Pt. 19)**:**3237–3248.
106. **Kaur, R., B. Ma, and B. P. Cormack.** 2007. A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc. Natl. Acad. Sci. USA* **104:**7628–7633.
107. **Kempf, M., J. Cottin, P. Licznar, C. Lefrancois, R. Robert, and V. Apaire-Marchais.** 2009. Disruption of the GPI protein-encoding gene IFF4 of *Candida albicans* results in decreased adherence and virulence. *Mycopathologia* **168:**73–77.
108. **Kingsbury, J. M., and J. H. McCusker.** 2008. Threonine biosynthetic genes are essential in *Cryptococcus neoformans*. *Microbiology* **154:**2767–2775.
109. **Kingsbury, J. M., and J. H. McCusker.** 2010. Cytocidal amino acid starvation of *Saccharomyces cerevisiae* and *Candida albicans* acetolactate synthase (*ilv2*Δ) mutants is influenced by the carbon source and rapamycin. *Microbiology* **156:**929–939.
110. **Kingsbury, J. M., Z. Yang, T. M. Ganous, G. M. Cox, and J. H. McCusker.** 2004. *Cryptococcus neoformans* Ilv2p confers resistance to sulfometuron methyl and is required for survival at 37°C and *in vivo*. *Microbiology* **150:**1547–1558.
111. **Kingsbury, J. M., Z. Yang, T. M. Ganous, G. M. Cox, and J. H. McCusker.** 2004. Novel chimeric spermidine synthase-saccharopine dehydrogenase gene (SPE3-LYS9) in the human pathogen *Cryptococcus neoformans*. *Eukaryot. Cell* **3:**752–763.
112. **Kirsch, D. R., and R. R. Whitney.** 1991. Pathogenicity of *Candida albicans* auxotrophic mutants in experimental infections. *Infect. Immun.* **59:**3297–3300.
113. **Klengel, T., W. J. Liang, J. Chaloupka, C. Ruoff, K. Schroppel, J. R. Naglik, S. E. Eckert, E. G. Mogensen, K. Haynes, M. F. Tuite, L. R. Levin, J. Buck, and F. A. Muhlschlegel.** 2005. Fungal adenylyl cyclase integrates CO_2 sensing with cAMP signaling and virulence. *Curr. Biol.* **15:**2021–2026.
114. **Klotz, S. A., D. J. Drutz, J. L. Harrison, and M. Huppert.** 1983. Adherence and penetration of vascular endothelium by *Candida* yeasts. *Infect. Immun.* **42:**374–384.
115. **Knechtle, P., S. Goyard, S. Brachat, O. Ibrahim-Granet, and C. d'Enfert.** 2005. Phosphatidylinositol-dependent phospholipases C Plc2 and Plc3 of *Candida albicans* are dispensable for morphogenesis and host-pathogen interaction. *Res. Microbiol.* **156:**822–829.
116. **Kunze, D., I. Melzer, D. Bennett, D. Sanglard, D. MacCallum, J. Norskau, D. C. Coleman, F. C. Odds, W. Schafer, and B. Hube.** 2005. Functional analysis of the phospholipase C gene CaPLC1 and two unusual phospholipase C genes, CaPLC2 and CaPLC3, of *Candida albicans*. *Microbiology* **151:**3381–3394.
117. **Kusch, H., S. Engelmann, D. Albrecht, J. Morschhauser, and M. Hecker.** 2007. Proteomic analysis of the oxidative stress response in *Candida albicans*. *Proteomics* **7:**686–697.
118. **Laforce-Nesbitt, S. S., M. A. Sullivan, L. L. Hoyer, and J. M. Bliss.** 2008. Inhibition of *Candida albicans* adhesion by recombinant human antibody single-chain variable fragment specific for Als3p. *FEMS Immunol. Med. Microbiol.* **54:**195–202.
119. **Lamarre, C., J. D. LeMay, N. Deslauriers, and Y. Bourbonnais.** 2001. *Candida albicans* expresses an unusual cytoplasmic manganese-containing superoxide dismutase (SOD3 gene product) upon the entry and during the stationary phase. *J. Biol. Chem.* **276:**43784–43791.
120. **Lay, J., L. K. Henry, J. Clifford, Y. Koltin, C. E. Bulawa, and J. M. Becker.** 1998. Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. *Infect. Immun.* **66:**5301–5306.
121. **Leidich, S. D., A. S. Ibrahim, Y. Fu, A. Koul, C. Jessup, J. Vitullo, W. Fonzi, F. Mirbod, S. Nakashima, Y. Nozawa, and M. A. Ghannoum.** 1998. Cloning and disruption of caPLB1, a phospholipase B gene involved in the pathogenicity of *Candida albicans*. *J. Biol. Chem.* **273:**26078–26086.
122. **Lermann, U., and J. Morschhauser.** 2008. Secreted aspartic proteases are not required for invasion of reconstituted human epithelia by *Candida albicans*. *Microbiology* **154:**3281–3295.
123. **Levitin, A., and M. Whiteway.** 2007. The effect of prostaglandin E2 on transcriptional responses of *Candida albicans*. *Microbiol. Res.* **162:**201–210.
124. **Liu, X. F., I. Elashvili, E. B. Gralla, J. S. Valentine, P. Lapinskas, and V. C. Culotta.** 1992. Yeast lacking superoxide dismutase. Isolation of genetic suppressors. *J. Biol. Chem.* **267:**18298–18302.
125. **Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90:**939–949.
126. **Loaiza-Loeza, S., B. Parra-Ortega, J. C. Cancino-Diaz, B. Illades-Aguiar, C. H. Hernandez-Rodriguez, and L. Villa-Tanaca.** 2009. Differential expression of *Candida dubliniensis*-secreted aspartyl proteinase genes (CdSAP1-4) under different physiological conditions and during infection of a keratinocyte culture. *FEMS Immunol. Med. Microbiol.* **56:**212–222.
127. **Lohse, M. B., and A. D. Johnson.** 2008. Differential phagocytosis of white versus opaque *Candida albicans* by *Drosophila* and mouse phagocytes. *PLoS One* **3:**e1473.
128. **Longo, V. D., E. B. Gralla, and J. S. Valentine.** 1996. Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species in vivo. *J. Biol. Chem.* **271:**12275–12280.
129. **Longo, V. D., L. L. Liou, J. S. Valentine, and E. B. Gralla.** 1999. Mitochondrial superoxide decreases yeast survival in stationary phase. *Arch. Biochem. Biophys.* **365:**131–142.
130. **Lorenz, M. C., J. A. Bender, and G. R. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot. Cell* **3:**1076–1087.
131. **Lorenz, M. C., and G. R. Fink.** 2001. The glyoxylate cycle is required for fungal virulence. *Nature* **412:**83–86.
132. **Loza, L., Y. Fu, A. S. Ibrahim, D. C. Sheppard, S. G. Filler, and J. E. Edwards, Jr.** 2004. Functional analysis of the *Candida albicans* ALS1 gene product. *Yeast* **21:** 473–482.
133. **Luo, S., S. Poltermann, A. Kunert, S. Rupp, and P. F. Zipfel.** 2009. Immune evasion of the human pathogenic yeast *Candida albicans*: Pra1 is a factor H, FHL-1 and plasminogen binding surface protein. *Mol. Immunol.* **47:** 541–550.
134. **Maidan, M. M., L. De Rop, M. Relloso, R. Diez-Orejas, J. M. Thevelein, and P. Van Dijck.** 2008. Combined inactivation of the *Candida albicans GPR1* and *TPS2* genes results in avirulence in a mouse model for systemic infection. *Infect. Immun.* **76:**1686–1694.
135. **Marcil, A., C. Gadoury, J. Ash, J. Zhang, A. Nantel, and M. Whiteway.** 2008. Analysis of PRA1 and its relationship to *Candida albicans*-macrophage interactions. *Infect. Immun.* **76:**4345–4358.
136. **Martchenko, M., A. M. Alarco, D. Harcus, and M. Whiteway.** 2004. Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. *Mol. Biol. Cell* **15:**456–467.

137. **Martinez-Esparza, M., A. Aguinaga, P. Gonzalez-Parraga, P. Garcia-Penarrubia, T. Jouault, and J. C. Arguelles.** 2007. Role of trehalose in resistance to macrophage killing: study with a *tps1/tps1* trehalose-deficient mutant of *Candida albicans*. *Clin. Microbiol. Infect.* **13:** 384–394.
138. **Martinez-Esparza, M., E. Martinez-Vicente, P. Gonzalez-Parraga, J. M. Ros, P. Garcia-Penarrubia, and J. C. Arguelles.** 2009. Role of trehalose-6P phosphatase (TPS2) in stress tolerance and resistance to macrophage killing in *Candida albicans*. *Int. J. Med. Microbiol.* **299:**453–464.
139. **Mor, N., and M. B. Goren.** 1987. Discrepancy in assessment of phagosome-lysosome fusion with two lysosomal markers in murine macrophages infected with *Candida albicans*. *Infect. Immun.* **55:**1663–1667.
140. **Moran, G. P., D. M. MacCallum, M. J. Spiering, D. C. Coleman, and D. J. Sullivan.** 2007. Differential regulation of the transcriptional repressor NRG1 accounts for altered host-cell interactions in *Candida albicans* and *Candida dubliniensis*. *Mol. Microbiol.* **66:**915–929.
141. **Moreno-Ruiz, E., M. Galan-Diez, W. Zhu, E. Fernandez-Ruiz, C. d'Enfert, S. G. Filler, P. Cossart, and E. Veiga.** 2009. *Candida albicans* internalization by host cells is mediated by a clathrin-dependent mechanism. *Cell. Microbiol.* **11:**1179–1189.
142. **Murad, A. M., P. Leng, M. Straffon, J. Wishart, S. Macaskill, D. MacCallum, N. Schnell, D. Talibi, D. Marechal, F. Tekaia, C. d'Enfert, C. Gaillardin, F. C. Odds, and A. J. Brown.** 2001. *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. *EMBO J.* **20:**4742–4752.
143. **Nadir, E., and M. Kaufshtein.** 2005. Images in clinical medicine. *Candida albicans* in a peripheral-blood smear. *N. Engl. J. Med.* **353:**e9.
144. **Nagahashi, S., T. Mio, N. Ono, T. Yamada-Okabe, M. Arisawa, H. Bussey, and H. Yamada-Okabe.** 1998. Isolation of *CaSLN1* and *CaNIK1*, the genes for osmosensing histidine kinase homologues, from the pathogenic fungus *Candida albicans*. *Microbiology* **144**(Pt. 2)**:** 425–432.
145. **Naglik, J. R., S. J. Challacombe, and B. Hube.** 2003. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev.* **67:**400–428.
146. **Naglik, J. R., C. A. Rodgers, P. J. Shirlaw, J. L. Dobbie, L. L. Fernandes-Naglik, D. Greenspan, N. Agabian, and S. J. Challacombe.** 2003. Differential expression of *Candida albicans* secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. *J. Infect. Dis.* **188:**469–479.
147. **Nakagawa, Y.** 2008. Catalase gene disruptant of the human pathogenic yeast *Candida albicans* is defective in hyphal growth, and a catalase-specific inhibitor can suppress hyphal growth of wild-type cells. *Microbiol. Immunol.* **52:**16–24.
148. **Nakagawa, Y., T. Kanbe, and I. Mizuguchi.** 2003. Disruption of the human pathogenic yeast *Candida albicans* catalase gene decreases survival in mouse-model infection and elevates susceptibility to higher temperature and to detergents. *Microbiol. Immunol.* **47:**395–403.
149. **Nantel, A., D. Dignard, C. Bachewich, D. Harcus, A. Marcil, A. P. Bouin, C. W. Sensen, H. Hogues, M. van het Hoog, P. Gordon, T. Rigby, F. Benoit, D. C. Tessier, D. Y. Thomas, and M. Whiteway.** 2002. Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol. Biol. Cell* **13:**3452–3465.
150. **Nasution, O., K. Srinivasa, M. Kim, Y. J. Kim, W. Kim, W. Jeong, and W. Choi.** 2008. Hydrogen peroxide induces hyphal differentiation in *Candida albicans*. *Eukaryot. Cell* **7:**2008–2011.
151. **Navarathna, D. H., K. W. Nickerson, G. E. Duhamel, T. R. Jerrels, and T. M. Petro.** 2007. Exogenous farnesol interferes with the normal progression of cytokine expression during candidiasis in a mouse model. *Infect. Immun.* **75:**4006–4011.
152. **Nazi, I., A. Scott, A. Sham, L. Rossi, P. R. Williamson, J. W. Kronstad, and G. D. Wright.** 2007. Role of homoserine transacetylase as a new target for antifungal agents. *Antimicrob. Agents Chemother.* **51:**1731–1736.
153. **Netea, M. G., K. Gijzen, N. Coolen, I. Verschueren, C. Figdor, J. W. Van der Meer, R. Torensma, and B. J. Kullberg.** 2004. Human dendritic cells are less potent at killing *Candida albicans* than both monocytes and macrophages. *Microbes Infect.* **6:**985–989.
154. **Nett, J. E., A. J. Lepak, K. Marchillo, and D. R. Andes.** 2009. Time course global gene expression analysis of an in vivo *Candida* biofilm. *J. Infect. Dis.* **200:**307–313.
155. **Newman, S. L., B. Bhugra, A. Holly, and R. E. Morris.** 2005. Enhanced killing of *Candida albicans* by human macrophages adherent to type 1 collagen matrices via induction of phagolysosomal fusion. *Infect. Immun.* **73:** 770–777.
156. **Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell.** 2006. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation in vitro and in vivo. *PLoS Pathog.* **2:**e63.
157. **Nobile, C. J., and A. P. Mitchell.** 2006. Genetics and genomics of *Candida albicans* biofilm formation. *Cell. Microbiol.* **8:**1382–1391.
158. **Noble, S. M., and A. D. Johnson.** 2005. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot. Cell* **4:**298–309.
159. **Oh, S. H., G. Cheng, J. A. Nuessen, R. Jajko, K. M. Yeater, X. Zhao, C. Pujol, D. R. Soll, and L. L. Hoyer.** 2005. Functional specificity of *Candida albicans* Als3p proteins and clade specificity of ALS3 alleles discriminated by the number of copies of the tandem repeat sequence in the central domain. *Microbiology* **151:** 673–681.
160. **Otoo, H. N., K. G. Lee, W. Qiu, and P. N. Lipke.** 2008. *Candida albicans* Als adhesins have conserved amyloid-forming sequences. *Eukaryot. Cell* **7:**776–782.
161. **Palmer, G. E., D. S. Askew, and P. R. Williamson.** 2008. The diverse roles of autophagy in medically important fungi. *Autophagy* **4:**982–988.
162. **Palmer, G. E., M. N. Kelly, and J. E. Sturtevant.** 2007. Autophagy in the pathogen *Candida albicans*. *Microbiology* **153:**51–58.
163. **Park, H., Y. Liu, N. Solis, J. Spotkov, J. Hamaker, J. R. Blankenship, M. R. Yeaman, A. P. Mitchell, H. Liu, and S. G. Filler.** 2009. Transcriptional responses of *Candida albicans* to epithelial and endothelial cells. *Eukaryot. Cell* **8:**1498–1510.
164. **Park, H., C. L. Myers, D. C. Sheppard, Q. T. Phan, A. A. Sanchez, J. E. Edwards, and S. G. Filler.** 2005. Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. *Cell. Microbiol.* **7:**499–510.
165. **Pascon, R. C., T. M. Ganous, J. M. Kingsbury, G. M. Cox, and J. H. McCusker.** 2004. *Cryptococcus neoformans* methionine synthase: expression analysis and requirement for virulence. *Microbiology* **150:**3013–3023.
166. **Phan, Q. T., P. H. Belanger, and S. G. Filler.** 2000. Role of hyphal formation in interactions of *Candida albicans* with endothelial cells. *Infect. Immun.* **68:**3485–3490.
167. **Phan, Q. T., R. A. Fratti, N. V. Prasadarao, J. E. Edwards, Jr., and S. G. Filler.** 2005. N-cadherin mediates

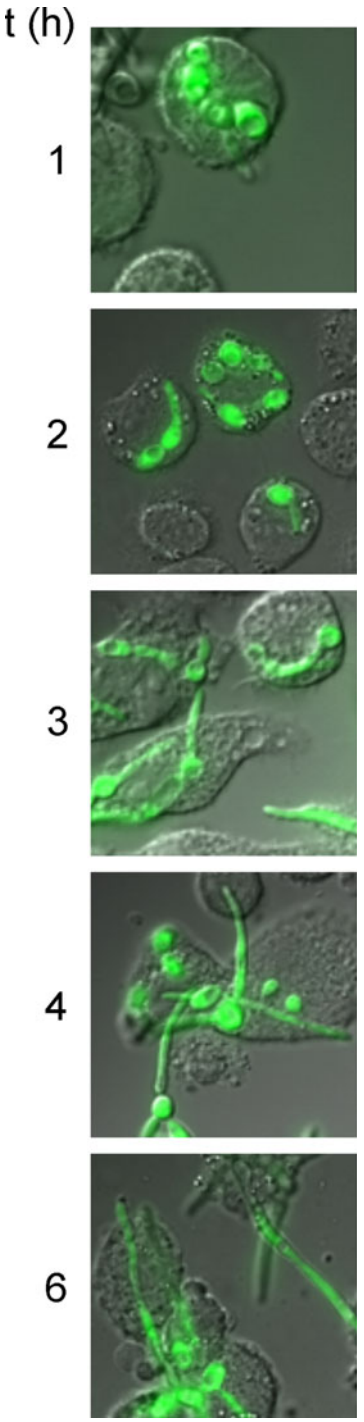
endocytosis of *Candida albicans* by endothelial cells. *J. Biol. Chem.* **280:**10455–10461.

168. **Phan, Q. T., C. L. Myers, Y. Fu, D. C. Sheppard, M. R. Yeaman, W. H. Welch, A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol.* **5:**e64.
169. **Piekarska, K., G. Hardy, E. Mol, J. van den Burg, K. Strijbis, C. van Roermund, M. van den Berg, and B. Distel.** 2008. The activity of the glyoxylate cycle in peroxisomes of *Candida albicans* depends on a functional beta-oxidation pathway: evidence for reduced metabolite transport across the peroxisomal membrane. *Microbiology* **154:**3061–3072.
170. **Piekarska, K., E. Mol, M. van den Berg, G. Hardy, J. van den Burg, C. van Roermund, D. Maccallum, F. Odds, and B. Distel.** 2006. Peroxisomal fatty acid β-oxidation is not essential for virulence of *Candida albicans*. *Eukaryot. Cell* **5:**1847–1856.
171. **Poltermann, S., A. Kunert, M. von der Heide, R. Eck, A. Hartmann, and P. F. Zipfel.** 2007. Gpm1p is a factor H-, FHL-1-, and plasminogen-binding surface protein of *Candida albicans*. *J. Biol. Chem.* **282:** 37537–37544.
172. **Ponniah, G., C. Rollenhagen, Y. S. Bahn, J. F. Staab, and P. Sundstrom.** 2007. State of differentiation defines buccal epithelial cell affinity for cross-linking to *Candida albicans* Hwp1. *J. Oral Pathol. Med.* **36:**456–467.
173. **Prigneau, O., A. Porta, J. A. Poudrier, S. Colonna-Romano, T. Noel, and B. Maresca.** 2003. Genes involved in beta-oxidation, energy metabolism and glyoxylate cycle are induced by *Candida albicans* during macrophage infection. *Yeast* **20:**723–730.
174. **Ramirez, M. A., and M. C. Lorenz.** 2007. Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot. Cell* **6:**280–290.
175. **Rauceo, J. M., R. De Armond, H. Otoo, P. C. Kahn, S. A. Klotz, N. K. Gaur, and P. N. Lipke.** 2006. Threonine-rich repeats increase fibronectin binding in the *Candida albicans* adhesin Als5p. *Eukaryot. Cell* **5:** 1664–1673.
176. **Ray, T. L., and C. D. Payne.** 1988. Scanning electron microscopy of epidermal adherence and cavitation in murine candidiasis: a role for *Candida* acid proteinase. *Infect. Immun.* **56:**1942–1949.
177. **Reggiori, F., and D. J. Klionsky.** 2002. Autophagy in the eukaryotic cell. *Eukaryot. Cell* **1:**11–21.
178. **Renna, M. S., S. G. Correa, C. Porporatto, C. M. Figueredo, M. P. Aoki, M. G. Paraje, and C. E. Sotomayor.** 2006. Hepatocellular apoptosis during *Candida albicans* colonization: involvement of TNF-alpha and infiltrating Fas-L positive lymphocytes. *Int. Immunol.* **18:** 1719–1728.
179. **Rennemeier, C., T. Frambach, F. Hennicke, J. Dietl, and P. Staib.** 2009. Microbial quorum-sensing molecules induce acrosome loss and cell death in human spermatozoa. *Infect. Immun.* **77:**4990–4997.
180. **Rhie, G. E., C. S. Hwang, M. J. Brady, S. T. Kim, Y. R. Kim, W. K. Huh, Y. U. Baek, B. H. Lee, J. S. Lee, and S. O. Kang.** 1999. Manganese-containing superoxide dismutase and its gene from *Candida albicans*. *Biochim. Biophys. Acta* **1426:**409–419.
181. **Richard, M. L., and A. Plaine.** 2007. Comprehensive analysis of glycosylphosphatidylinositol-anchored proteins in *Candida albicans*. *Eukaryot. Cell* **6:**119–133.
182. **Rivas, V., and T. J. Rogers.** 1983. Studies on the cellular nature of *Candida albicans*-induced suppression. *J. Immunol.* **130:**376–379.
183. **Roetzer, A., N. Gratz, P. Kovarik, and C. Schuller.** 2010. Autophagy supports *Candida glabrata* survival during phagocytosis. *Cell. Microbiol.* **12:**199–216.
184. **Rogers, T. J., and E. Balish.** 1978. Effect of systemic candidiasis on blastogenesis of lymphocytes from germfree and conventional rats. *Infect. Immun.* **20:**142–150.
185. **Rogers, T. J., and E. Balish.** 1978. Suppression of lymphocyte blastogenesis by *Candida albicans*. *Clin. Immunol. Immunopathol.* **10:**298–305.
186. **Rotrosen, D., J. E. Edwards, Jr., T. R. Gibson, J. C. Moore, A. H. Cohen, and I. Green.** 1985. Adherence of *Candida* to cultured vascular endothelial cells: mechanisms of attachment and endothelial cell penetration. *J. Infect. Dis.* **152:**1264–1274.
187. **Rotstein, D., J. Parodo, R. Taneja, and J. C. Marshall.** 2000. Phagocytosis of *Candida albicans* induces apoptosis of human neutrophils. *Shock* **14:**278–283.
188. **Rubin-Bejerano, I., I. Fraser, P. Grisafi, and G. R. Fink.** 2003. Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **100:** 11007–11012.
189. **Sanchez, A. A., D. A. Johnston, C. Myers, J. E. Edwards, Jr., A. P. Mitchell, and S. G. Filler.** 2004. Relationship between *Candida albicans* virulence during experimental hematogenously disseminated infection and endothelial cell damage in vitro. *Infect. Immun.* **72:** 598–601.
190. **Sandovsky-Losica, H., N. Chauhan, R. Calderone, and E. Segal.** 2006. Gene transcription studies of *Candida albicans* following infection of HEp2 epithelial cells. *Med. Mycol.* **44:**329–334.
191. **Sanglard, D., B. Hube, M. Monod, F. C. Odds, and N. A. Gow.** 1997. A triple deletion of the secreted aspartyl proteinase genes *SAP4*, *SAP5*, and *SAP6* of *Candida albicans* causes attenuated virulence. *Infect. Immun.* **65:** 3539–3546.
192. **Sato, T., K. Iwabuchi, I. Nagaoka, Y. Adachi, N. Ohno, H. Tamura, K. Seyama, Y. Fukuchi, H. Nakayama, F. Yoshizaki, K. Takamori, and H. Ogawa.** 2006. Induction of human neutrophil chemotaxis by *Candida albicans*-derived beta-1,6-long glycoside side-chain-branched beta-glucan. *J. Leukoc. Biol.* **80:**204–211.
193. **Saville, S. P., A. L. Lazzell, C. Monteagudo, and J. L. Lopez-Ribot.** 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot. Cell* **2:**1053–1060.
194. **Schaller, M., M. Bein, H. C. Korting, S. Baur, G. Hamm, M. Monod, S. Beinhauer, and B. Hube.** 2003. The secreted aspartyl proteinases Sap1 and Sap2 cause tissue damage in an in vitro model of vaginal candidiasis based on reconstituted human vaginal epithelium. *Infect. Immun.* **71:**3227–3234.
195. **Schaller, M., C. Borelli, H. C. Korting, and B. Hube.** 2005. Hydrolytic enzymes as virulence factors of *Candida albicans*. *Mycoses* **48:**365–377.
196. **Schaller, M., H. C. Korting, W. Schafer, J. Bastert, W. Chen, and B. Hube.** 1999. Secreted aspartic proteinase (Sap) activity contributes to tissue damage in a model of human oral candidosis. *Mol. Microbiol.* **34:**169–180.
197. **Schaller, M., and G. Weindl.** 2009. Models of oral and vaginal candidiasis based on in vitro reconstituted human epithelia for the study of host-pathogen interactions. *Methods Mol. Biol.* **470:**327–345.
198. **Schaller, M., K. Zakikhany, J. R. Naglik, G. Weindl, and B. Hube.** 2006. Models of oral and vaginal candidiasis based on in vitro reconstituted human epithelia. *Nat. Protoc.* **1:**2767–2773.

199. **Scheper, M. A., M. E. Shirtliff, T. F. Meiller, B. M. Peters, and M. A. Jabra-Rizk.** 2008. Farnesol, a fungal quorum-sensing molecule triggers apoptosis in human oral squamous carcinoma cells. *Neoplasia* **10:**954–963.
200. **Schroppel, K., M. Kryk, M. Herrmann, E. Leberer, M. Rollinghoff, and C. Bogdan.** 2001. Suppression of type 2 NO-synthase activity in macrophages by *Candida albicans*. *Int. J. Med. Microbiol.* **290:**659–668.
201. **Sentandreu, M., M. V. Elorza, R. Sentandreu, and W. A. Fonzi.** 1998. Cloning and characterization of *PRA1*, a gene encoding a novel pH-regulated antigen of *Candida albicans*. *J. Bacteriol.* **180:**282–289.
202. **Sharkey, L. L., W. L. Liao, A. K. Ghosh, and W. A. Fonzi.** 2005. Flanking direct repeats of *his*G alter *URA3* marker expression at the *HWP1* locus of *Candida albicans*. *Microbiology* **151:**1061–1071.
203. **Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu, A. S. Ibrahim, S. G. Filler, M. Zhang, A. J. Waring, and J. E. Edwards, Jr.** 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. *J. Biol. Chem.* **279:**30480–30489.
204. **Shin, D. H., S. Jung, S. J. Park, Y. J. Kim, J. M. Ahn, W. Kim, and W. Choi.** 2005. Characterization of thiol-specific antioxidant 1 (TSA1) of *Candida albicans*. *Yeast* **22:**907–918.
205. **Slekar, K. H., D. J. Kosman, and V. C. Culotta.** 1996. The yeast copper/zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection. *J. Biol. Chem.* **271:**28831–28836.
206. **Smith, D. A., S. Nicholls, B. A. Morgan, A. J. Brown, and J. Quinn.** 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. *Mol. Biol. Cell* **15:**4179–4190.
207. **Soloviev, D. A., W. A. Fonzi, R. Sentandreu, E. Pluskota, C. B. Forsyth, S. Yadav, and E. F. Plow.** 2007. Identification of pH-regulated antigen 1 released from *Candida albicans* as the major ligand for leukocyte integrin alphaMbeta2. *J. Immunol.* **178:**2038–2046.
208. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283:**1535–1538.
209. **Staib, P., M. Kretschmar, T. Nichterlein, G. Kohler, S. Michel, H. Hof, J. Hacker, and J. Morschhauser.** 1999. Host-induced, stage-specific virulence gene activation in *Candida albicans* during infection. *Mol. Microbiol.* **32:** 533–546.
210. **Stanley, V. C., and R. Hurley.** 1969. The growth of *Candida* species in cultures of mouse peritoneal macrophages. *J. Pathol.* **97:**357–366.
211. **Stehr, F., A. Felk, A. Gacser, M. Kretschmar, B. Mahnss, K. Neuber, B. Hube, and W. Schafer.** 2004. Expression analysis of the *Candida albicans* lipase gene family during experimental infections and in patient samples. *FEMS Yeast Res.* **4:**401–408.
212. **Strijbis, K., C. W. van Roermund, W. F. Visser, E. C. Mol, J. van den Burg, D. M. MacCallum, F. C. Odds, E. Paramonova, B. P. Krom, and B. Distel.** 2008. Carnitine-dependent transport of acetyl coenzyme A in *Candida albicans* is essential for growth on nonfermentable carbon sources and contributes to biofilm formation. *Eukaryot. Cell* **7:**610–618.
213. **Sundstrom, P.** 2002. Adhesion in *Candida* spp. *Cell. Microbiol.* **4:**461–469.
214. **Sundstrom, P., J. E. Cutler, and J. F. Staab.** 2002. Re-evaluation of the role of *HWP1* in systemic candidiasis by use of *Candida albicans* strains with selectable marker *URA3* targeted to the *ENO1* locus. *Infect. Immun.* **70:** 3281–3283.
215. **Szabo, I., L. Guan, and T. J. Rogers.** 1995. Modulation of macrophage phagocytic activity by cell wall components of *Candida albicans*. *Cell. Immunol.* **164:**182–188.
216. **Theiss, S., G. Ishdorj, A. Brenot, M. Kretschmar, C. Y. Lan, T. Nichterlein, J. Hacker, S. Nigam, N. Agabian, and G. A. Kohler.** 2006. Inactivation of the phospholipase B gene *PLB5* in wild-type *Candida albicans* reduces cell-associated phospholipase A2 activity and attenuates virulence. *Int. J. Med. Microbiol.* **296:**405–420.
217. **Thewes, S., M. Kretschmar, H. Park, M. Schaller, S. G. Filler, and B. Hube.** 2007. In vivo and ex vivo comparative transcriptional profiling of invasive and non-invasive *Candida albicans* isolates identifies genes associated with tissue invasion. *Mol. Microbiol.* **63:**1606–1628.
218. **Ullmann, B. D., H. Myers, W. Chiranand, A. L. Lazzell, Q. Zhao, L. A. Vega, J. L. Lopez-Ribot, P. R. Gardner, and M. C. Gustin.** 2004. Inducible defense mechanism against nitric oxide in *Candida albicans*. *Eukaryot. Cell* **3:**715–723.
219. **Urban, C., K. Sohn, F. Lottspeich, H. Brunner, and S. Rupp.** 2003. Identification of cell surface determinants in *Candida albicans* reveals Tsa1p, a protein differentially localized in the cell. *FEBS Lett.* **544:**228–235.
220. **Urban, C., X. Xiong, K. Sohn, K. Schroppel, H. Brunner, and S. Rupp.** 2005. The moonlighting protein Tsa1p is implicated in oxidative stress response and in cell wall biogenesis in *Candida albicans*. *Mol. Microbiol.* **57:** 1318–1341.
221. **van Loon, A. P., B. Pesold-Hurt, and G. Schatz.** 1986. A yeast mutant lacking mitochondrial manganese-superoxide dismutase is hypersensitive to oxygen. *Proc. Natl. Acad. Sci. USA* **83:**3820–3824.
222. **Vergne, I., J. Chua, S. B. Singh, and V. Deretic.** 2004. Cell biology of *Mycobacterium tuberculosis* phagosome. *Annu. Rev. Cell Dev. Biol.* **20:**367–394.
223. **Vylkova, S., W. S. Jang, W. Li, N. Nayyar, and M. Edgerton.** 2007. Histatin 5 initiates osmotic stress response in *Candida albicans* via activation of the Hog1 mitogen-activated protein kinase pathway. *Eukaryot. Cell* **6:** 1876–1888.
224. **Walker, L. A., D. M. Maccallum, G. Bertram, N. A. Gow, F. C. Odds, and A. J. Brown.** 2009. Genome-wide analysis of *Candida albicans* gene expression patterns during infection of the mammalian kidney. *Fungal Genet. Biol.* **46:**210–219.
225. **Wellington, M., K. Dolan, and D. J. Krysan.** 2009. Live *Candida albicans* suppresses production of reactive oxygen species in phagocytes. *Infect. Immun.* **77:**405–413.
226. **White, S. J., A. Rosenbach, P. Lephart, D. Nguyen, A. Benjamin, S. Tzipori, M. Whiteway, J. Mecsas, and C. A. Kumamoto.** 2007. Self-regulation of *Candida albicans* population size during GI colonization. *PLoS Pathog.* **3:**e184.
227. **Wysong, D. R., L. Christin, A. M. Sugar, P. W. Robbins, and R. D. Diamond.** 1998. Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. *Infect. Immun.* **66:**1953–1961.
228. **Yang, Z., R. C. Pascon, A. Alspaugh, G. M. Cox, and J. H. McCusker.** 2002. Molecular and genetic analysis of the *Cryptococcus neoformans MET3* gene and a *met3* mutant. *Microbiology* **148:**2617–2625.
229. **Zakikhany, K., J. R. Naglik, A. Schmidt-Westhausen, G. Holland, M. Schaller, and B. Hube.** 2007. In vivo transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. *Cell. Microbiol.* **9:**2938–2954.
230. **Zaragoza, O., M. A. Blazquez, and C. Gancedo.** 1998. Disruption of the *Candida albicans TPS1* gene encoding trehalose-6-phosphate synthase impairs formation of

hyphae and decreases infectivity. *J. Bacteriol.* **180:** 3809–3815.

231. **Zaragoza, O., C. de Virgilio, J. Ponton, and C. Gancedo.** 2002. Disruption in *Candida albicans* of the *TPS2* gene encoding trehalose-6-phosphate phosphatase affects cell integrity and decreases infectivity. *Microbiology* **148:** 1281–1290.
232. **Zhang, X., M. De Micheli, S. T. Coleman, D. Sanglard, and W. S. Moye-Rowley.** 2000. Analysis of the oxidative stress regulation of the *Candida albicans* transcription factor, Cap1p. *Mol. Microbiol.* **36:**618–629.
233. **Zhao, X., S. H. Oh, G. Cheng, C. B. Green, J. A. Nuessen, K. Yeater, R. P. Leng, A. J. Brown, and L. L. Hoyer.** 2004. *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. *Microbiology* **150:** 2415–2428.
234. **Zhao, X., S. H. Oh, and L. L. Hoyer.** 2007. Deletion of ALS5, ALS6 or ALS7 increases adhesion of *Candida albicans* to human vascular endothelial and buccal epithelial cells. *Med. Mycol.* **45:**429–434.
235. **Zhao, X., S. H. Oh, and L. L. Hoyer.** 2007. Unequal contribution of *ALS9* alleles to adhesion between *Candida albicans* and human vascular endothelial cells. *Microbiology* **153:**2342–2350.
236. **Zhao, X., S. H. Oh, K. M. Yeater, and L. L. Hoyer.** 2005. Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* **151:**1619–1630.
237. **Zhou, H., and M. C. Lorenz.** 2008. Carnitine acetyltransferases are required for growth on non-fermentable carbon sources but not for pathogenesis in *Candida albicans*. *Microbiology* **154:**500–509.
238. **Zhu, W., and S. G. Filler.** 2010. Interactions of *Candida albicans* with epithelial cells. *Cell. Microbiol.* **12:**273–282.
239. **Zink, S., T. Nass, P. Rosen, and J. F. Ernst.** 1996. Migration of the fungal pathogen *Candida albicans* across endothelial monolayers. *Infect. Immun.* **64:**5085–5091.
240. **Znaidi, S., K. S. Barker, S. Weber, A. M. Alarco, T. T. Liu, G. Boucher, P. D. Rogers, and M. Raymond.** 2009. Identification of the *Candida albicans* Cap1p regulon. *Eukaryot. Cell* **8:**806–820.



COLOR PLATE 5 (CHAPTER 17) C. *albicans* morphogenesis and escape following macrophage phagocytosis. A wild-type C. *albicans* strain expressing a constitutive ACT1 promoter-GFP construct was incubated with J774A.1 macrophages for the indicated times. Fluorescent images are overlaid onto the phase image to highlight the fungal cells. 10.1128/9781555817176.ch17cp5

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch18

18

Gene Expression during the Distinct Stages of Candidiasis

DUNCAN WILSON, FRANCOIS MAYER, AND BERNHARD HUBE

Candida albicans is a remarkably versatile pathogen, capable of causing infections of virtually every organ of the human host, and a frequent cause of multiple organ failure and death in hospitalized patients. Damage of epithelial barriers or a weakened immune system may pave the way for *C. albicans* to spread from mucosal surfaces throughout the body. Since few microbial pathogens are able to proliferate at so many different body sites, the question arises of how this fungus may have gained its potential to survive at multiple body sites, to evade elimination by the immune system, and to cause disease. During evolution, *C. albicans* has found its ecological niche as a benign colonizer, a commensal of mucosal surfaces and skin, occasionally causing superficial infections. Life-threatening systemic candidiasis has emerged as a significant medical problem only in the past few decades, and it seems unlikely that the fungus has gained distinct fitness and virulence attributes necessary for this type of infection during this period. Rather, *C. albicans* must have already possessed the factors required to cause systemic disease before its emergence as an invasive pathogen. It is possible that during adaptation as a commensal organism, *C. albicans* has selected properties which may also be favorable for survival in a number of other microenvironments of the host. Indeed, even during commensal carriage, *C. albicans* represents a highly versatile organism, as it is a successful colonizer of the urogenitary tract, skin, oral cavity, and gastrointestinal (GI) tract. These body sites are environments of widely varying conditions—in terms of environmental stresses, pH, volatile gases (e.g., O_2 and CO_2), humidity, and nutrient availability—yet *C. albicans* successfully adheres to these various surfaces (epidermis, enterocytes, and urogenitary and oral epithelium, either directly or via keratinized layers, extracellular matrix proteins, or other members of the microbial flora), resists environmental stresses, avoids or evades aggressive immune responses, and acquires sufficient nutrients to proliferate. Therefore, its commensal lifestyle must have adequately prepared the fungus for life in deeper tissue (40). These four aspects (colonization, stress tolerance, immune evasion, and nutrient acquisition) represent common themes, probably necessary in all niches and stages of candidiasis (Fig. 1). As discussed below, this opportunistic pathogen expresses sometimes different sets of genes to perform these tasks (Table 1). In this chapter we summarize some of the known and proposed infection strategies and underlying transcriptional responses which govern them. Although similar strategies may be used by other pathogenic *Candida* species which are commonly found as commensals (*C. dubliniensis*, *C. parapsilosis*, and *C. glabrata*), we focus on *C. albicans* as the most common and best-investigated species.

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COLONIZATION OF MUCOSAL SURFACES

The overwhelming source of all *C. albicans* infections (both superficial and systemic) is of endogenous origin, either from biofilms associated with indwelling medical devices or from epithelial tissues. The ability of *C. albicans* to colonize surfaces therefore constitutes a vital first step in many forms of candidiasis. Since biofilm formation is described in chapter 19, here we focus on interactions with mucosal surfaces. In this section we discuss the strategies employed, from adhesion to tissue destruction, during colonization or infection of three distinct mucosal niches—the GI tract, the oral cavity, and the urogenitary tract—believed to be the major sources of disseminated, oral, and vaginal candidiasis, respectively. It should be mentioned that *C. albicans* can also colonize and infect the skin, particularly in infants or elderly people; however, due to the scarcity of gene expression data on skin colonization and infection, this niche is not discussed here.

Commensalism in the GI Tract

As described below ("Dissemination"), several routes exist for *C. albicans* to reach deeper tissue and cause disseminated candidiasis (52). It is generally believed that the main reservoir for *C. albicans* in humans is the GI tract and that systemic infections predominantly originate from this reservoir (65, 103). For example, surgical patients undergoing recurrent GI perforation are at an increased risk of *Candida*

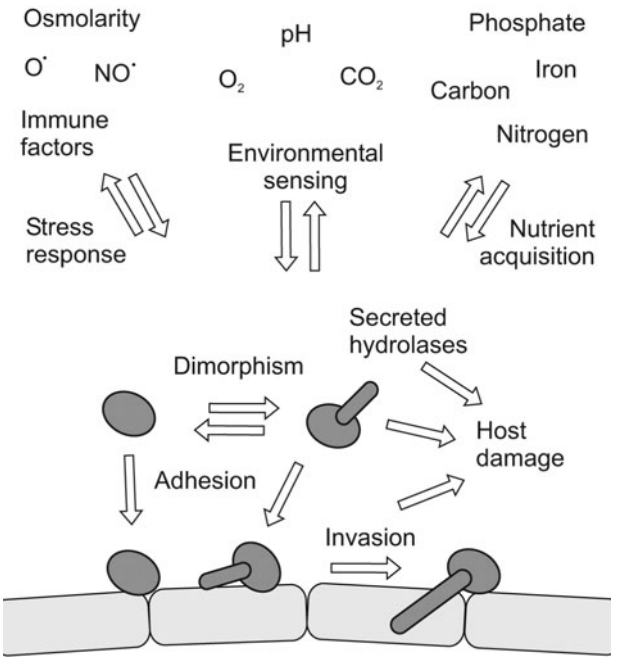

FIGURE 1 General aspects of *Candida albicans*-host interactions. During all forms of candidiasis, the fungus must sense and respond to the local environment, acquire nutrients, and resist environmental stresses. C. *albicans* is capable of adhering to various substrata, growing in different morphologies (dimorphism), invading host cells, and secreting extracellular hydrolases which, under certain circumstances, can lead to damage of the host. For a more detailed summary of the genes involved in these processes during the distinct stages of candidiasis, refer to Table 1. 10.1128/9781555817176.ch18f1

peritonitis, and a GI source is most common in neutropenic patients suffering from candidemia (67).

Despite this, relatively few studies have dealt with interactions of C. *albicans* with cells or components of the GI tract, and fewer still have dealt with the commensal lifestyle of C. *albicans*. One of the reasons for this is the difficulty in establishing commensal models in commonly used in vivo systems. In nature, the GI tracts of neither rats nor mice are colonized by C. *albicans* (14, 21, 35, 100), and in order to establish colonization, some form of treatment such as the use of antibiotics is usually necessary (74, 78, 101, 102).

One such study (87) analyzed the expression of the *LIP* gene family (encoding secreted lipases) during alimentary tract colonization. Schofield et al. reported that while *LIP4* to *-8* were constitutively expressed in all studied areas of the alimentary tract, *LIP1* to *-3* and *LIP9* expression was specific to the GI tract and not observed in oral tissue. Moreover, of these GI tract-expressed genes, constitutive expression was restricted to the gastric tissue (*LIP1*, *-3*, and *-9*) or the cecum contents (*LIP2*). This provides an example of differential expression of a gene family during passage of C. *albicans* through the alimentary tract.

At the time of publication of this book, global gene expression studies of commensal colonization of the GI tract by C. *albicans* had not been performed. The laboratory of Kumamoto, however, has analyzed the expression profile of selected genes by quantitative reverse transcriptase PCR of C. *albicans* cells colonizing the large intestine of a gnotobiotic piglet and from the cecum and ileum of mice (106). In this study, White et al. demonstrated a novel role for Efh1 in the commensal lifestyle of C. *albicans*. The gene *EFH1* represents a paralogue of the well-known, and extensively studied, gene *EFG1*, encoding a key transcriptional regulator of morphology; however, while *EFG1* has been demonstrated to play major roles in a number of biological processes (including metabolism, chlamydospore formation, the yeast-to-hypha transition, and expression of hypha-associated genes) and is required for virulence, the function of Efh1 had remained rather more elusive, as an *efh1*Δ mutant displayed no obvious in vitro defects (19). Paradoxically, although *EFH1* was more highly expressed during GI tract colonization, an *efh1*Δ mutant displayed higher levels of colonization. Furthermore, overexpression of *EFH1* reduced colonization levels. The authors concluded that Efh1 is involved in negative self-regulation of C. *albicans* population size during GI tract colonization. It would appear, therefore, that maintenance of the commensal state is at least partially dependent on direct activity of C. *albicans*. This in itself is an intriguing finding. Because a fully intact immune system, together with a normal microbial flora, is sufficient to fully protect the individual from all disease state manifestations of candidiasis, it had widely been presumed that the host environment itself determined the fundamental balance between commensalism and pathogenicity (39). However, this discovery of a fungus-driven activity which negatively regulates population size in vivo suggests that C. *albicans* perhaps plays a more active role in promoting the commensal relationship than previously thought. From an evolutionary perspective, this is not particularly surprising: for the most part, C. *albicans* is a more successful commensal organism than pathogen; consequently, it would appear that the fungus has developed mechanisms to persist in its ecological niche without irritating or damaging the host. In the future it will be crucial to use technologies such as genome-wide analysis of commensal phases of C. *albicans* to uncover fungal activities which contribute to either the commensal or the pathogenic lifestyle, or both, of C. *albicans*.

White et al. (106) also made a number of interesting observations that have challenged certain other ideas regarding in vivo gene expression. Firstly, it was shown that although the majority of cells colonizing the GI tract were of yeast morphology, a number of genes which had previously been described as hypha specific (or at least hypha associated) were transcriptionally upregulated in the GI tract, including *ECE1*, *RBT1*, and *RBT4*. Moreover, these three genes were also shown to be expressed in the GI tract at relatively high levels by a mutant lacking the transcriptional regulators *EFG1* and *CPH1*. In vitro, the expression of these three genes is *EFG1* dependent. Indeed, under most studied in vitro conditions, *EFG1* is required for both hyphal growth and expression of hypha-associated genes, as well as the correct expression of numerous metabolic genes (19).

Therefore, during in vivo commensal-phase colonization of the GI tract, there would appear to be an uncoupling of (conventionally described) gene expression from morphology and regulatory networks. Possible clues as to the reason for the observed uncoupling of gene expression from Efg1-mediated morphogenesis in fact came from the same laboratory in earlier studies. Giusani et al. (32) demonstrated that under microaerophilic conditions, Efg1 actually acts as a repressor of hyphal development. The group of Ernst continued work on unraveling the Efg1 network, initially finding that while the expression of numerous hypha-associated and metabolic genes relied on Efg1 under normoxia (19), under

TABLE 1 Summary of transcriptional profiling of *C. albicans* infection model studies[a]

Host niche	Infection model(s)	Transcriptional response	Genes of interest	Reference
Oral tissue	Human patient samples; RHE	Alternative carbon utilization[a]; nitrogen acquisition[b]; nitrosative stress[c]; phosphate uptake[d]; hypha-associated[e]; pH response[f]; adhesion[g]; invasion[h]; secreted hydrolases[i]	(*MAL31*, *PCK1*, *MLS1*, *ICL1*, *FOX3*, *PXA1*, *FOX2*)[a]; (*GNP1*, *CAR1*, *CAR2*)[b]; (*YHB5*, *SSU1*, *YHB1*)[c]; *PHO84*[d]; (*FKH1*, *ATP2*, *TEF1*, *SOD5*, *RBT1*, *ECE1*, *HYR1*)[e]; (*PHR1*, *PRA1*)[e,f]; *ALS3*[e,g,h]; *HWP1*[e,g]; *SAP5*[e,i]	109
Gastrointestinal tract	In vivo piglet and mouse intestine	NA	*EFH1*, *YHB5*, *ECE1*, *RBT1*, *RBT4*	106
Gastrointestinal tract	Cell culture monolayers	Cell surface proteins/adhesion	*PRA1*, *PGA7*, *PGA23*, *HWP1*	90
Vagina	Cell culture monolayers	Cell surface proteins/adhesion	*PRA1*, *PGA7*, *PGA23*, *HWP1*	90
Neutrophils	Ex vivo human blood	Alternative carbon utilization[a]; nitrogen acquisition[b]; oxidative stress[j]	(*ICL1*, *MLS1*)[a]; (*MEP2*, *MEP3*, *GCN4*)[b]; (*CTA1*, *TRX1*)[j]	28, 76
Macrophages	Cell culture	Alternative carbon utilization[a]; nitrogen acquisition[c]	(*FBP1*, *PCK1*, *ICL1*, *MLS1*, *ACO1*, *CIT1*)[a]; (*OPT1*, *-4*, *-9*, *PTR2*)[c]	49
Endothelium	Cell culture monolayers	Cell surface remodeling	*PMT1*, *AMS1*, *CHT2*, *-3*, *ERG4*	68
Kidney	In vivo rabbit kidney	Alternative carbon source assimilation[a]; phosphate uptake[d]; adhesion[g]; stress adaptation[k]	(*ACO1*, *ACS1*, *CIT1*, *FAA4*, *MLS1*, *POX4*, *SDH12*)[a]; *PHO84*[d];(*ALS1*, *ALS2*, *ALS4*)[g]; (*CTA1*, *ENA22*)[k]	4, 105
Liver	In vivo mouse and ex vivo pig liver	Nitrogen acquisition[b]; phosphate uptake[d]; hypha-associated[e]; pH response[f]; adhesion[g]; invasion[h]; secreted hydrolases[i]; iron acquisition[l]; thermal stress[m]; carbohydrate metabolism[n]	*SAP2*[b,i]; *PHO84*[d]; *PHR1*[e,f]; *HWP1*[e,g]; *SAP5,6*[e,i]; *ALS3*[e,g,h]; (*SOD5*, *ECE1*)[e]; (*FTR1*, *FRP1*, *CFL1*, *FET34*, *CTR1*, *RBT5*)[l]; (*HSP78*, *HSP90*, *HSP104*, *HSP12*, *HSP70*)[m]; (*PFK1*, *PDA1*, *PDX1*, *KGD1*, *KGD2*)[n]	98

[a]For distinct stages of candidiasis (host niche), infection models have been used to monitor expression patterns indicative of particular activities and processes (transcriptional response) based on the transcriptional upregulation of certain genes (genes of interest). Where multiple transcriptional responses are listed, superscript letters in the third and fourth columns link the biological processes or activities with the relevant upregulated genes. Note that some genes are involved in more than one process or activity. NA, not applicable.

hypoxia, Efg1 functioned as a repressor of filamentation and a positive regulator of genes involved in fatty acid biosynthesis (89). However, even this reversal in Efg1 function under hypoxia does not hold true for all filamentation-associated processes. For example, during biofilm formation under hypoxic conditions, Efg1 is required for the expression of biofilm "hallmark" genes, including those involved in glycolysis, sulfur metabolism, and the antioxidative response (see chapter 19). On the other hand, the transcriptional regulators Tec1, Ace2, and Czf1 were shown to be required for biofilm formation under normoxic, but not hypoxic, conditions (94).

What is therefore becoming clear, from these and other studies, is that regulatory networks in *C. albicans* (even those which were thought to be relatively well described) can have very different functions upon alteration of a single environmental factor (in this case, oxygen levels). Going back to the situation in the GI tract, could it be that the inherent anaerobic/microaerophilic nature of this anatomical niche is one of the factors contributing to Efg1-independent expression of hypha-associated genes by yeast cells? Supporting this view, Sosinska et al. (91) identified expression (this time at the protein level) of Hwp1 and Als3 on the surface of yeast cells growing under hypoxic conditions, together with iron limitation in a vagina-simulative in vitro model. The genes encoding these two proteins were previously described as hypha specific, as their expression was restricted to the hyphal morphology in vitro (5). Therefore, an increasing body of in vivo data, as well as carefully designed in vitro experiments that reflect specific aspects of distinct anatomical niches, is beginning to challenge some of the accepted conventions of *C. albicans* gene expression.

Colonization and Infection of Oral Epithelium

The mucosal epithelium of the oral cavity represents one of the first layers of living host cells to encounter potentially harmful microorganisms. In healthy hosts, *C. albicans* often colonizes this niche as part of the normal microbial flora, which consists mainly of bacteria. Colonization of nonpathogenic probiotic microbes provides a barrier effect and is believed to protect the host from attack by exogenous but also opportunistic endogenous pathogenic microorganisms. Despite the importance of commensal carriage, gene expression studies of this stage in the lifestyle of *C. albicans* have not been carried out as yet, and even defined animal models for asymptomatic carriage have not been adequately developed (see above).

Certain predisposing conditions permit *C. albicans* to switch from a harmless commensal of the oral mucosa to an aggressive pathogen able to cause superficial infections of the oral cavity and oropharyngeal regions. In response to harmful microorganisms, a healthy oral epithelium produces

and secretes a multitude of antimicrobial molecules and stimulates the immune system in order to counteract a potential infection. Conditions which favor the opportunistic transition of *C. albicans* from a benign oral colonizer to a pathogen include imbalances or defects in the immune system. The weakened immune system of human immunodeficiency virus (HIV)-infected patients, for example, strongly favors the pathogenesis of oral candidiasis, and this is reflected by the fact that as many as 90% of HIV-positive individuals developed oral candidiasis before the advent of highly active antiretroviral therapy (Ruhnke, 2002). Interestingly, however, HIV patients, including those suffering from oral candidiasis, very rarely develop deep-seated disseminated infections (see below). Therefore, although oral candidiasis represents a significant disease manifestation in its own right, it does not appear to represent a significant predisposing factor for systemic candidiasis.

Elucidating *C. albicans*-oral epithelium interactions is of central interest to understand how the fungus can breach and damage this first human line of defense. Although mouse models for oral infections exist (26), a significant drawback of these models is the fact that specific features of the oral epithelium are fundamentally different between humans and mice; for example, the level of cornification is significantly higher in mouse than in human oral epithelial tissues. Schaller et al. (85, 86) addressed this problem by developing a model of oral candidiasis based on in vitro-reconstituted human epithelia (RHE). These bioengineered tissues mimic certain aspects of the in vivo human tissue. For example, the oral tissue produces key immune effector molecules and displays tissue repair mechanisms, paralleling the in vivo situation. Culture monolayers of oral cells represent simplified versions of the RHE model, with only one confluent layer of host cells. The oral head and neck squamous cell carcinoma-derived cell line TR146, the human epithelial cell line FaDu from a squamous cell carcinoma of the hypopharynx, and the immortalized human oral keratinocyte cell line OKF6/TERT-2 are the predominant cell types used in these models (20, 68, 86). Finally, investigations of biopsy samples from human patients suffering from oral candidiasis perhaps represent the best source for gaining insight into the infection process occurring in vivo. However, few studies have been performed using patient samples due to ethical reasons or challenges in obtaining sufficient fungal biomass for analysis.

Zakikhany et al. (109) examined the different stages of oral candidiasis using the RHE model. Moreover, the authors went on to analyze the transcriptional profile of *C. albicans* during the various stages of interaction with oral tissue (below) and compared this to gene expression data of clinical samples obtained from HIV-positive patients suffering from oral candidiasis. The authors were able to define three relatively distinct stages of the RHE infection process: an early attachment phase, a middle invasion phase, and a late tissue destruction phase.

Initial colonization, whether during commensalism or infection, requires adhesion to the host cells and is believed to be mediated by fungal adhesins, including members of the agglutinin-like sequence (Als) family and hyphal wall protein 1 (Hwp1), which had been shown to be expressed in vivo (33, 60, 109). Sandovsky-Losica et al. (80) demonstrated upregulation of the adhesin-encoding genes *ALS2* and *ALS5* during coincubation with HEp-2 cells. Using the RHE model, attachment of *C. albicans* to the oral epithelium was shown to occur within the first 3 h of the interaction, and the process of attachment itself stimulated hyphal formation with a concomitant upregulation of hypha-associated genes such as *HWP1*, *FKH1* (encoding a forkhead transcription factor which regulates morphogenesis) (9), *ATP2* (encoding the predicted F1 beta subunit of F_1F_0 ATPase complex) (95), *ALS3* (encoding an adhesin, invasin, and ferritin receptor) (2, 37, 72), *SOD5* (encoding a copper- and zinc-containing superoxide dismutase) (28, 51), *RBT1* (encoding a cell wall protein with similarity to Hwp1) (12), *ECE1* (encoding a secreted protein of poorly defined function) (11), and *HYR1* (encoding a glycosylphosphatidylinositol [GPI]-anchored predicted cell wall protein) (6). Following on from this study, Park et al. (68) used monolayers of FaDu oral epithelial cells to investigate gene expression during the early phase of *C. albicans*-oral epithelial interactions (45, 90, and 180 min). These authors did not report a significant enrichment of gene ontology (GO) categories in the genes which were upregulated on oral epithelial cells, perhaps due to the relatively low number of significantly upregulated genes (only 47 genes over all three time points). Moreover, over 40% of the induced genes were of unknown function. Of the 47 oral epithelial induced genes reported by Park et al. (68), only 3 were also significantly upregulated during oral RHE infection (109). Remarkably, these three genes are all putatively involved in amino acid biosynthesis and/or biofilm formation (31). The three commonly upregulated genes were *TRP1*, encoding a phosphoribosylanthranilate isomerase, essential for tryptophan biosynthesis (66); *MET1*, encoding a putative uroporphyrin-3 *C*-methyltransferase, essential for methionine biosynthesis; and *SEC14*, the product of which is an essential phospholipid transfer protein involved in membrane trafficking and the production of secretory vesicles from the Golgi apparatus (55).

Although Park et al. (68) did not report an overrepresentation in GO categories for the genes upregulated upon contact with epithelial cells, three functional categories were significantly enriched within the downregulated genes. These were ribonucleoprotein complex biogenesis, protein catabolic processes, and adhesion. The authors conclude that the first two categories—decreased protein synthesis coupled with decreased protein catabolism—may reflect a response to stress. Indeed, ribosome biogenesis is known to be downregulated after phagocytosis by macrophages (49) and in response to nitric oxide (38), and one of the defense mechanisms of macrophages and epithelial cells in response to infection is the production of nitric oxide. Because of the common reference used in this study (cells growing on polystyrene at 37°C, a condition which induces hyphal development), the observed enrichment of downregulated genes involved in adhesion may be due to two reasons: genes involved in adhesion (including *ALS3* and *HWP1*) are genuinely downregulated in response to oral epithelial cells, or these genes are induced on both oral epithelial cells and plastic, but induction on plastic is stronger than on oral epithelial cells. Three lines of evidence point to the latter explanation: (i) other microarray studies have demonstrated an upregulation of genes involved in adhesion upon interaction with epithelial cells (90, 104a, 109), (ii) *C. albicans* generally adheres more efficiently to plastic surfaces than to cell culture monolayers (our unpublished data), and (iii) a number of the (downregulated) adhesin genes (e.g., *ALS3* and *HWP1*) have been demonstrated to be critical for adhesion to oral epithelial cells (92, 104a, 110). However, because different oral cell types were used among these studies, it cannot be excluded that these adhesins are downregulated on certain types of oral cells.

All the studies described here, however, represent situations in which *C. albicans* behaves as an aggressive pathogen. To our knowledge, no studies have so far been undertaken to address gene expression during the harmless commensal stage of *C. albicans* colonization of the oral cavity. This is, we believe, one of the most important next steps for understanding the molecular mechanisms which account for the pathogenesis of oral candidiasis. Ideally, an in vivo model of commensal oral *C. albicans* carriage should be established, whereby a disease manifestation (oral candidiasis) can be experimentally induced (perhaps via immunosuppression or removal of a protective bacterial flora). The commensally colonized tissue could then be monitored to detect the natural fluctuations in *C. albicans* gene expression in the oral cavity. Simultaneously, the transcript profile of *C. albicans* in the disease state could be determined over time. A parallel time course study such as this would provide extremely important information on oral candidiasis: will *C. albicans* employ the same gene products during commensal and infectious growth of the same body site (niche associated), or will certain infection-specific factors be expressed in the disease phase but not the commensal phase (39)? It is likely that at least some genes involved in the interaction between *C. albicans* and host epithelial cells have similar functions in both the commensal and the pathogenic stages. For example, adhesion is a prerequisite for colonization and pathogenesis, and even superficial invasion via induced endocytosis (see below) may contribute to both, commensal colonization by supporting attachment to mucosal surfaces and pathogenesis of superficial infection and damage of epithelial cells.

Infection of Vaginal Epithelium

The importance of *C. albicans* as a human pathogen is reflected by the fact that up to 75% of all women worldwide suffer at least once in their lifetime from vulvovaginal candidiasis, an infection of the vaginal mucosa (24). A minority (5 to 8%) of these women develop recurrent infections (25). In 70 to 90% of all cases, vulvovaginal candidiasis is caused by *C. albicans* (69).

Since the vagina forms a discrete niche inside the human body with an acidic pH, it is likely that pH-regulated fungal genes are involved in the colonization and infection process. In *C. albicans*, the response to changes in environmental pH is governed by the Rim101 (regulator of IME2) pathway (75). De Bernardis and colleagues (18) proposed in 1998 that the pH of the host niche controls gene expression and virulence of *C. albicans*. In order to determine if *C. albicans* responds to the pH of the host niche, the authors investigated the virulence of strains with mutations in *PHR1* and *PHR2*, two pH-regulated genes (56, 81). *PHR1* is expressed under alkaline or neutral conditions, while *PHR2* is induced under acidic conditions (56, 81). Both genes encode GPI-anchored glycosidases important for cell wall integrity (27). A rat vaginitis model and a model of candidemia were used for these experiments and revealed two interesting observations. In the vaginitis model, the mutant lacking the acidic pH-induced gene, *PHR2*, had reduced virulence, while the *phr1*Δ mutant was indistinguishable from a wild-type strain. Conversely, the mutant lacking the neutral/alkaline pH-induced gene, *PHR1*, was avirulent in a mouse model of systemic candidiasis, while the *phr2*Δ mutant was fully virulent. The conclusion from these observations is that *PHR1* and *PHR2* encode proteins that are functionally exchangeable but differentially expressed depending on the environmental pH and possibly optimized for different pH ranges. The fact that *C. albicans* possesses two functionally interchangeable proteins expressed during, and required for, distinct stages of candidiasis underscores the remarkable adaptability of this fungus.

Other studies have aimed to understand the biological principles of interactions of *C. albicans* with vaginal epithelial cells and to identify genes expressed during these interactions. Like the RHE model mimicking in vivo oral epithelial cells, an in vitro model has been established for investigating the interaction of *C. albicans* with vaginal cells (86). In this in vitro model of vaginal candidiasis the authors studied the expression and role of secreted aspartic proteases (Saps) during infection and tissue damage of vaginal epithelium. The expression of several *SAP* genes was detected, and mutants lacking *SAP1* or *SAP2* had a reduced potential to cause tissue damage. *SAP* expression was also observed in samples isolated from patients with vaginal candidiasis.

The expression profile of *SAP* genes in the course of vaginal infection in mice was also analyzed by Taylor et al. (97). Using recombination-based in vivo expression technology and green fluorescent protein-expressing *C. albicans* reporter strains, the authors identified expression of *SAP4* and *SAP5*, both of which are hypha-associated genes. One possible drawback of this study may be the fact that the pH values of mouse vaginal tissues are rather neutral, which may influence *C. albicans* morphology, *SAP* expression, and Sap activity. *SAP* genes, in particular *SAP2*, have also been shown to be involved in vaginal candidiasis of rats (17). Most members of the Sap isoenzyme family have a pH optimum in the acidic pH range (41, 57, 58) and are likely to be active in the vaginal niche. In fact, Naglik et al. (59) have shown by quantitative reverse transcriptase PCR that *SAP* genes are expressed in human vaginal candidiasis, in particular *SAP5* and *SAP9*.

In conclusion, pH appears to be an important environmental cue for the vaginopathic potential of *C. albicans*. However, it is unlikely that pH is the only factor influencing the specific process of vaginal infections. Temperature, hypoxia, and iron limitation are other important cues associated with vaginal candidiasis, and several studies have investigated the in vitro transcriptional profile of *C. albicans* in response to these environmental conditions (45, 63, 89).

So far, only cell culture monolayers have been used to monitor the genome-wide transcriptional profile of *C. albicans* during interactions with vaginal cells, and preliminary data have been published (90). The transcription levels of genes such as *PRA1* (encoding a pH-regulated immunogenic cell surface antigen), *PGA7* (encoding a protein described as a putative precursor of a hyphal surface antigen), *PGA23* (encoding a putative GPI-anchored protein of unknown function), and *HWP1* (see above) were shown to be upregulated in *C. albicans* cells attached to vaginal cells in comparison to suspension cultures. Certainly, more studies investigating the transcriptional dynamics of *C. albicans* are desirable in order to understand the fine-tuning in regulation of gene expression under vaginopathic conditions.

INVASION AT MUCOSAL SITES

As mentioned above, superficial infections with *C. albicans* are extremely common. In these types of infections, *C. albicans* invades and damages superficial layers of epithelial tissues. However, as discussed in the following sections, in certain cases *C. albicans* can also penetrate deeper tissues, pass the endothelial cell layer, and reach the bloodstream. Infections with *C. albicans* then can become life-threatening as

the fungus manages to infect virtually every human organ. A crucial step between colonization and disseminated infections with C. *albicans* is the invasion into deeper tissue layers.

Invasion of Epithelium

The RHE model (see above) has also been used to study the stages of epithelial invasion and tissue destruction as a paradigm of deeper infection. In the oral RHE model, the invasion phase (3 to 12 h postinfection) temporally follows the attachment phase (discussed above). The invasion phase is characterized by extensive hyphal growth and two distinct invasion routes, induced endocytosis and active penetration of the epithelium (16, 109, 115). Induced endocytosis is a host-driven process triggered by a microbial invasin. Zhu and Filler (115) were the first to show that the hypha-associated GPI-anchored protein Als3 plays a key role in uptake by epithelial cells via the host receptor E-cadherin. In fact, *ALS3* was shown to be expressed during experimental oral infection of RHE and in patient samples (109). The process of active penetration is less well studied and is thought to be mediated via a combination of mechanical pressure from the extending hyphal tip and the secretion of hydrolytic enzymes, such as proteases, lipases, or phospholipases, which would assist in host tissue degradation and deeper invasion. In the RHE model of the secreted aspartic protease genes, only *SAP5* was significantly upregulated during the invasion phase. However, *LIP1*, *LIP8*, and *LIP9* were all significantly induced, suggesting that lipid degradation may play an important role in the invasion process. Finally, *PLB1*, encoding the main secreted phospholipase of C. *albicans*, was not significantly upregulated. Using mutants lacking *SAP* genes or the *PLB1* gene, Dalle et al. (16) showed that *SAP1* to *-3* and *SAP4* to *-6* are possibly involved in both active penetration and induced endocytosis, while *PLB1* was dispensable for invasion. Furthermore, lipase inhibitors did not influence the invasion capacity of C. *albicans*.

In line with the observed morphology, numerous hypha-associated genes were transcriptionally upregulated during invasion of the oral RHE, including *ALS3*, *HWP1*, *SOD5*, *ECE1*, and *RBT1*. Of note, despite extensive invasion in the mid-phase (3 to 12 h postinfection), substantial damage of the oral RHE tissue did not occur until the late phase (12 to 24 h). Although tissue invasion and tissue damage are not entirely independent events during oral infection, invasion via induced endocytosis can occur without causing any damage. In fact, even killed hyphae are taken up by epithelial cells without any sign of epithelial damage (16, 26). Therefore, fungal factors and viability are essential for damage but not invasion.

Following invasion, the subsequent tissue destruction stage (12 to 24 h) in the RHE model was associated with a plethora of adaptive responses (indicated at the transcriptional level), presumably reflecting the rapidly altering environment generated by disruption of host cells. Fungal cells adapt to an increase in pH at this stage, reflected by the upregulation of the neutral/alkaline pH marker genes, *PHR1* and *PRA1*, and *RIM101*, encoding a key alkaline-pH-responsive transcription factor (see above). Although the observed induction of neutral/alkaline pH genes may have been influenced by exposure of the invaded fungal cells to the cell culture maintenance medium in the abluminal compartment of the RHE system, it likely reflects a genuine aspect of this form of infection, as these three genes were also upregulated in samples from patients suffering from oral candidiasis.

At the late phase of oral infection, the transcriptional profile of C. *albicans* suggested that fungal cells faced a glucose-poor environment. *ICL1* and *MLS1*, which encode the two key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthase, respectively, were strongly transcriptionally upregulated at 12 and 24 h postinfection. *PCK1*, encoding a key enzyme of gluconeogenesis, was also strongly induced. In addition, the observed upregulation of members of the *LIP* gene family (see above) may have resulted in the release of lipids as potential carbon sources. This would explain why the genes *FOX2* (encoding a 3-hydroxyacyl-coenzyme A [CoA] epimerase), *FOX3* (peroxisomal 3-oxoacyl CoA thiolase), and *PXA1* (putative peroxisomal, half-size adrenoleukodystrophy protein), all potentially involved in fatty acid β-oxidation, were also induced in the late phase.

Presumably to gain access to nitrogen, several genes involved in amino acid sensing and transport (*GNP1*, *CAR1*, and *CAR2*) were transcriptionally upregulated, although there did not appear to be a significant response to amino acid starvation. Despite this, as mentioned above, a number of genes involved in methionine biosynthesis, including *MET1* (putatively involved in sulfate assimilation and methionine biosynthesis) and *HAL21* (involved in sulfur recycling), were upregulated during RHE infection, perhaps pointing either to a scarcity of sulfur-containing amino acids during oral infection or to a higher demand for these molecules by the fungus during oral candidiasis. However, it should be noted that the wholesale upregulation of genes of the methionine biosynthesis pathway, as was observed upon exposure to human blood, was not detected (28, 76).

Interestingly, the gene encoding the high-affinity phosphate transporter *PHO84* was induced during the phase when oral tissue damage was strongest, suggestive of low environmental levels of phosphate. This is of note because, as we will see in the following sections, *PHO84* is upregulated during multiple infection types (even among studies which employed different control conditions). This may indicate that the ability to scavenge phosphate in the host environment represents a challenge during the distinct stages of candidiasis or that *PHO84* has other functions necessary in the host environment.

Apart from the limitation of certain nutrients, the only other clear stress condition encountered by the fungus appeared to be nitrosative stress, as indicated by the upregulation of the marker genes *YHB5*, *SSU1*, and *YHB1*, involved in detoxification of nitric oxide (38). It is known that epithelial cells produce nitrogen monoxide radicals as part of their innate immune response against microorganisms, and it would therefore appear that the experimental RHE model is capable of mounting at least this innate defense mechanism against C. *albicans* infection.

In the recent investigations of Zakikhany et al. (109) and Park et al. (68), a large number of genes found to be upregulated upon contact with epithelial cells had not been previously characterized. Zakikhany et al. (109) disrupted seven of these and found that at least three genes of unknown function were important for C. *albicans* to cause damage of epithelial cells. Park and coworkers (68) elucidated the function of three other genes of unknown function, *YCK2* (encoding a plasma membrane protein), *VPS51* (encoding a protein with a role in vacuolar function), and *UEC1* (encoding a unique protein required for resistance to cell membrane stress). Each of these genes was required for normal damage of epithelial cells and resistance to anti-

microbial peptides. These studies indicate that a number of genes of unknown function may exist that play distinct roles during interaction with epithelial cells.

In summary, genes expressed during mucosal infections reflect distinct activities of *C. albicans* which can be monitored and quantified, such as the yeast-to-hypha transition, adhesion, invasion, or damage. However, the expression profiles also indicate dynamic cellular adaptations to changing microenvironments such as changing pH values or alterations in accessible nutrients or other responses to stress, including stress possibly produced by host cell activities, such as nitrosative stress.

DISSEMINATION

Systemic candidiasis involves (i) entry of fungal cells into the bloodstream, (ii) survival within blood, (iii) exit from the bloodstream, and (iv) invasion of an internal organ(s). In this section, aspects of dissemination, from entry into to exit from the cardiovascular system and gene expression profiles associated with these stages, are described and discussed.

Circulatory System Access

As discussed in the previous sections, one way for *C. albicans* to reach the circulation is to invade at mucosal sites. However, this is by no means the only route of access (52). At least three other conceivable ways exist for fungal cells to reach the circulation: (i) epidermal disruption via severe burns or the insertion of medical devices such as catheters permits migration from the skin to the blood, (ii) abdominal surgery or polytrauma provides a route from the GI tract to the bloodstream, and (iii) dispersal events from biofilms growing on indwelling catheters directly seed the bloodstream. A detailed discussion on biofilm formation can be found in chapter 19.

Spread via the Bloodstream

Disseminated candidiasis relies on the spread of *C. albicans* throughout the body via the bloodstream. However, this is no mean feat, as the bloodstream contains numerous factors and immune effector cells which can kill almost every microorganism. Of key importance are professional phagocytic cells. Chapter 17 of this book describes the survival and escape strategies that *C. albicans* employs when faced with phagocytes.

Escape from the Bloodstream—Endothelial Interactions

Unlike certain specialized nonfungal eukaryotic human parasites (for example, *Plasmodium falciparum* or trypanosomes), *C. albicans* does not appear to reside in the circulatory system for extended periods, since inoculated cells of *C. albicans* cannot be reisolated from circulating blood within very short periods after experimental bloodstream infections (52). Frequently, the term clearance is used for this phenomenon. However, it is not clear whether these cells have been killed by immune cells, have attached to endothelial cells, or have escaped from the bloodstream and invaded into deeper tissue. Usually, at least 90% of inoculated cells (up to 99%) are trapped in the liver and in the lung 5 to 15 min after experimental animal infections (50). A large portion of the trapped cells is still viable after 15 min, and some cells are still in the bloodstream attached to endothelial cells, as shown by scanning electron microscopy of organ sections (50, 52, 84). However, after 5 to 10 h, circulating *C. albicans* cells are usually not recovered (50), suggesting that fungal cells are in fact cleared from the murine circulatory system. In order to infect internal (solid) organs during disseminated candidiasis, *C. albicans* must therefore escape the cardiovascular system. The major proposed mechanism for exit of fungal cells from the circulation is adhesion to and subsequent transmigration across the luminal endothelial lining of blood vessels of the microvascular system.

Adhesion to Endothelial Cells

A number of cell surface molecules have been demonstrated experimentally to confer adhesion to human endothelial cells in vitro. Deletion mutants of all eight members of the *ALS* gene family have been tested for endothelial adhesion capability (1, 30, 36, 110–114). Interestingly, deletion of either *ALS1*, *-2*, *-3*, *-4*, or *-9* attenuated adhesion of *C. albicans* to endothelial cells, suggesting that each of the corresponding adhesins may add to the adhesive properties of *C. albicans* during interaction with endothelial cells and that Als proteins may have synergistic functions. To more realistically simulate the bloodstream, the group of Thornhill as well as our own laboratory conducted studies on *C. albicans*-endothelial adhesion under flow conditions, which mimic the physical environment of the circulatory system. At low physical pressure (0.25 to 1.5 dynes/cm^2), Grubb et al. (34) demonstrated preferential adhesion of yeast over hyphal morphologies, providing evidence that disseminating yeast cells can efficiently adhere to endothelium at low physical pressures such as those found in postcapillary venules. These authors did not, however, observe adhesion at pressures above 3 dynes/cm^2. Indeed, at higher pressures, a specific stage in the yeast-to-hypha transition appears to be necessary for endothelial adhesion. Wilson and Hube (107) reported that germ tubes of a particular stage (3 to 7 μm) adhered more efficiently than both shorter and longer hyphal morphologies and that this process is governed by the hypha-specific G1 cyclin encoded by *HGC1*. Moreover, in both studies, the authors reported a directionality of germ tube adhesion under flow conditions, suggesting that the hyphal tip is the major anchor during dynamic endothelial adhesion. These studies indicate that adhesive and other virulence-related properties are not simply restricted to the expression of a set of stage-specific genes but also require a distinct localization of proteins, an optimal physical shape, and an overall optimal three-dimensional structure of the fungus.

Invasion into Endothelial Cells

Following adhesion, the fungus must next traverse the endothelium in order to gain access to deeper tissue. There are no studies which provide definite evidence of how *C. albicans* may traverse the endothelium in vivo, but a number of possible exit strategies have been discussed. These include (i) induced endocytosis, (ii) active penetration, (iii) inter-endothelial cell translocation, (iv) carriage by phagocytes, and (v) transmigration through endothelial fenestrations. Furthermore, the molecular mechanisms underlying most of these processes are as yet unclear. As with other stages of candidiasis, hyphal formation is expected to play a key role. However, Saville et al. (83) demonstrated that yeast cells were also able to leave the bloodstream and disseminate to organs. Nevertheless, yeast cells did not cause disease unless filamentation occurred, providing further evidence for the importance of invasive filamentous growth in causing

damage to the host (82). How yeast cells may traverse through the endothelial barrier remains unclear. Indeed, the best in vitro-studied endothelial invasion mechanisms, induced endocytosis, rely on hyphal formation. This is because the fungal adhesin which binds to endothelial cell N-cadherin and triggers endocytosis of fungal cells, Als3, is almost exclusively expressed on hyphal cells under most conditions. The role of Als3 as an invasin was convincingly demonstrated by the group of Scott Filler (72), which showed that (i) an *als*3Δ *C. albicans* mutant was unable to undergo induced endocytosis, (ii) heterologous expression of Als3 by *S. cerevisiae* stimulated endocytosis, and (iii) latex beads coated with recombinant Als3 were efficiently endocytosed by endothelial cells. However, as discussed above, the expression of hypha-associated genes may be uncoupled from morphology in vivo (91, 106), and other invasion mechanisms such as active penetration exist (16). In addition, *C. albicans* has been shown to transcytose across human endothelial cells without affecting monolayer integrity (42).

Gene Expression of *C. albicans* during Interaction with Endothelial Cells

As well as representing one of the most significant contributors towards elucidating the molecular mechanisms of *C. albicans*-endothelial interactions, Filler's group has also published the first genome-wide transcriptional profiling study of *C. albicans* in association with human endothelial cells (68). Although transcriptional profiling of *C. albicans* interacting with endothelial cells did not identify *ALS3* (or any of the other *ALS* gene family members) as upregulated, consideration of experimental design and careful examination of supplementary data suggest that *ALS3* may in fact be strongly induced on endothelial cells. The authors here normalized expression data against mRNA levels from *C. albicans* growing on polystyrene tissue culture plates in RPMI cell culture medium at 37°C in a 5% CO_2 atmosphere (arguably one of the strongest in vitro hypha induction protocols). Therefore, it is not surprising that hypha-associated genes, such as *ALS3*, were not defined as upregulated on endothelial cells. Under the same conditions, *ALS3* was defined as significantly (between 2.3- and 2.8-fold) downregulated at all time points on oral epithelial cells, but not on endothelial cells, suggesting that, relatively speaking, *ALS3* is more highly expressed on endothelial than oral epithelial cells. In addition, *ALS1* and *ALS9* were also both downregulated on oral epithelial but not endothelial cells, fitting with experimental data that these two genes encode proteins which mediate adhesion to endothelial cells (30, 112).

In addition to the importance of *ALS* gene family members, *C. albicans* appears to respond to the endothelial environment by structural remodeling of its cell surface. Among the (rather limited number of) significantly upregulated genes on endothelial cells were genes coding for a protein mannosyltransferase (*PMT1*), a β-mannosidase (*AMS1*), two chitinases (*CHT2* and *CHT3*), and a sterol reductase (*ERG4*) (68). Indeed, the glycosylation state of cell wall proteins is likely to play a major role, as a *pmt6*Δ mutant with defective O glycosylation of secreted proteins displays attenuated endothelial adhesion (99). Park et al. (68) also report significant upregulation of numerous genes of unknown function (on average, 38% of endothelial induced genes were uncharacterized), suggesting that many factors involved in endothelial interaction have yet to be characterized.

DEEP-SEATED ORGAN INFECTION

To study the virulence properties of *C. albicans* in a mouse model, two infection routes are commonly used. Intravenous infection results in direct hematogenous dissemination via the bloodstream. Target organ colonization by *C. albicans* following intravenous infection depends on the site of injection but usually affects most major organs. For example, following infection via the lateral tail vein, colonization of the brain, heart, spleen, and liver commonly occurs, but the major target organ following this route of infection is the kidney (52). Another method for inducing invasive candidiasis is via intraperitoneal infection. Following this route of infection, *C. albicans* primarily adheres to and invades the liver and other peritoneal organs, such as the pancreas or spleen. At later stages the fungus is able to disseminate hematogenously and reach the heart, kidneys, lungs, and brain.

In a third model, dissemination from colonization of the GI tracts of mice is induced by forms of immune suppression and/or GI damage (8, 15, 44, 79). However, as yet, no transcriptional profiling has been performed under such a condition.

In the following section we discuss the gene expression patterns employed by *C. albicans* during infection of two major target organs (kidneys and liver).

Gene Expression within Kidney Lesions

Similar to the observations made for mucosal infections (see above), many of the transcriptional activities associated with kidney infections reflect nutrient acquisition and stress response.

Nutrient Acquisition—Carbon

The first study to perform transcriptional profiling of *C. albicans* during an in vivo infection was that of Andes et al. (4). The authors investigated the mRNA levels of *C. albicans* from pooled murine kidneys and found that genes associated with glucose utilization were downregulated during growth in the kidneys. This early result turned out to set the trend for the majority of infection-related global gene expression studies. With the possible exception of liver infection (see below), microarray analyses have indicated that *C. albicans* faces a glucose-poor environment in most of the host niches studied to date and likely employs alternative carbon assimilation pathways. Following on from the work of Andes, the laboratory of Al Brown together with other members of the Aberdeen Fungal Group revisited *C. albicans* gene expression in the context of kidney lesions, this time using a rabbit infection model (105). In agreement with the earlier study, Walker et al. (105) detected differential expression of numerous genes associated with carbon assimilation. For example, the gene encoding the pyruvate kinase, which catalyzes the final—ATP-generating—step of glycolysis, *CDC19*, was downregulated in rabbit kidney lesions. Moreover, the genes encoding the hexokinase (*HXK2*) and three sugar transporters, known to be induced under high glucose concentrations (*HXT5*/*HGT8*, *HXT61*/*HGT6*, and *HXT62*/*HGT7*), were also all downregulated. Conversely, genes associated with the glyoxylate cycle (*ACO1*, *ACS1*, *CIT1*, and *SDH12*), including *MLS1*, which encodes the malate synthase, as well as genes involved in fatty acid β-oxidation (*FAA4* and *POX4*) were upregulated during growth in kidney lesions. Based on transcript levels alone, therefore, glucose would seem to be in short supply and *C. albicans* appears to utilize alternative carbon assimi-

lation pathways such as β-oxidation, the tricarboxylic acid and glyoxylate cycles, and gluconeogenesis during kidney infections. Even a when microarray result yields a relatively unambiguous indication of carbon assimilation pathways such as this (105), the true in vivo situation is probably far less simple, and care should be taken in data interpretation. For example, an earlier study from the same group utilized "single-cell profiling" (based on reporter strains) to investigate carbon assimilation by *C. albicans* during growth within kidney lesions (7). Here, the expression of a glyoxylate cycle (*ICL1*), a gluconeogenesis (*PCK1*), and two glycolytic (*PFK2*, *PYK1*/*CDC19*) marker genes was determined via fusion of the promoter of interest to a green fluorescent protein reporter. Using this approach it was determined that around half of the infecting cells expressed *ICL1* and a third expressed *PCK1*, suggesting that only subpopulations of cells utilize the glyoxylate cycle and gluconeogenesis within such kidney lesions. Surprisingly, both of the glycolysis marker genes, *PYK1*/*CDC19* and *PFK2*, were induced in virtually the entire population of cells infecting the kidney. This contradictory observation can perhaps be explained by the fact that both *PYK1*/*CDC19* and *PFK2* in *C. albicans* are induced not only in the presence of glucose but also during active growth in the presence of alternative carbon sources, such as amino acids (7).

Nitrogen Acquisition

To acquire nitrogen from protein, *C. albicans* possesses a family of *SAP* genes, the in vitro and in vivo regulation of which has been the subject of numerous studies (see also above) (58). Although none of the *SAP* genes were identified as upregulated during rabbit kidney infection by microarray analysis (probably due to the transient expression profiles of the various members), some clues as to the resultant available nitrogen source can be gleaned. For example, three amino acid permeases and an oligopeptide transporter were upregulated during kidney infection, suggestive of degraded protein products serving as a nitrogen source during kidney infection.

Iron and Phosphate Acquisition

Because the essential element iron is tightly sequestered within the host, efficient iron acquisition strategies represent a universal virulence factor of virtually all pathogenic microbes, and *C. albicans*, in this regard, is no exception. However, only a single gene annotated as involved in iron acquisition (*FRE30*/*FRE7*, which encodes the putative ferric reductase) was found to be upregulated in the kidneys in the study of Walker et al. (105). In addition, the important molecule phosphate is probably scavenged from the host environment, as reflected by the upregulation of *PHO84*, which encodes a high-affinity phosphate transporter (see above). The fact that only single genes involved in iron and phosphate uptake were identified as upregulated is at first perhaps surprising, as the control cells, bathed in cell culture media, have access to relatively high levels of free iron and phosphate. However, this observation comes into perspective when one considers the number of genes dedicated to such uptake systems available to the organism. The *C. albicans* genome contains at least 15 homologous genes encoding predicted ferric reductases and at least as many phosphate permeases. The elemental importance of gaining these nutrients has resulted in large gene families—each member subtly optimized for specific conditions and niches. Furthermore, *C. albicans* does not rely on the uptake of free iron only but has developed strategies to gain iron directly from human iron-containing proteins (3). It is therefore likely that the heterogeneous populations of cells growing within the kidney are expressing various and variable combinations of these genes—ironing out significant differential transcript levels.

Stress Response

As with probably every other host niche, *C. albicans* likely faces environmental stresses during growth within the kidney, in particular those cells facing infiltrating phagocytes. By published microarray analysis, however, this was only reflected in the upregulation of two stress-associated genes: those for a catalase (*CTA1*/*CAT1*) (10, 23, 61,108) and a putative sodium transporter (*ENA22*/*ENA2*) (10, 23, 46, 109). This is again most likely due to heterogeneous cell populations (niche-within-a-niche principle). Indeed, Enjalbert et al. (22) used single-cell profiling to analyze the oxidative stress response during growth within the kidneys. Placement of green fluorescent protein under the control of the *CTA1*, *TTR1*, and *TRX1* promoters followed by murine intravenous infection with the reporter strains and subsequent analysis of resulting kidney lesions demonstrated that as few as ~5% of *C. albicans* cells infecting the kidney were exposed to significant oxidative stress (equivalent to around 0.4 mM H_2O_2). It is possible that a similar (low) percentage of cells are exposed to significant oxidative stress in the rabbit kidney (105) and that this subpopulation generates significant levels of mRNA (compared to control cells) to be detected by microarray hybridization. Alternatively, inherent differences between mouse and rabbit kidneys (such as, for example, significantly different levels of neutrophil infiltration to the site of infection) may also be an explanation. So, although clearly important for surviving attack by neutrophils in the bloodstream, the true significance of the oxidative stress response in the progression of deep-seated infections remains elusive. What is clear is that *C. albicans* cells which infect kidney tissue are not exposed to uniform environmental conditions.

Other Factors

Because the expression data of Walker et al. (105) were normalized against mRNA obtained from cells growing in cell culture media at physiological temperature, the majority of known hypha-associated genes were not identified as upregulated; indeed, a subset consisting of *HYR1*, *SOD5*, *ECE1*, *ALS3*, *PHR1*, and *RBT5* were all described as downregulated. Analogous to the observed regulation of carbon assimilation genes, the "downregulation" of some of these genes was probably due to the growth stage of the cells, as well as to physical aspects of the surrounding environment. For example, actively growing (control) hyphal cells probably require higher levels of Sod5 to deal with endogenously produced oxygen radicals (88) than their counterparts in the kidney lesion. Another factor to consider is local environmental modification by the fungus itself. The neutral/alkaline pH marker gene *PHR1* is, very likely, upregulated in the neutral/alkaline pH of the RPMI cell culture medium. Although the native pH of the kidneys is probably quite similar, it is possible that *C. albicans* is capable of actively acidifying its local environment (2). If this is the case, it would certainly explain why this acidic pH-repressed gene was monitored as downregulated in this study.

Liver Invasion

To identify *C. albicans* genes that are expressed during invasion of the liver, Thewes et al. (98) used genome-wide

transcriptional profiling in vivo and ex vivo. By analyzing the different phases of intraperitoneal infection from attachment to tissue penetration in a time course experiment and by comparing the profile of an invasive strain with that of a noninvasive strain, Thewes et al. (98) identified genes and transcriptional patterns which are associated with the invasion process.

Nutrient Acquisition—Carbon

Probably the most striking aspect of liver infection (in relation to other studied stages of candidiasis) is the apparent abundance of sugars available to the pathogen during invasion of this organ. As noted in the above sections, virtually all microarray analyses of C. *albicans* performed thus far suggest that the fungus faces a glucose-poor environment and relies on alternative carbon assimilation pathways, such as β-oxidation, the glyoxylate cycle, and gluconeogenesis, during most forms of infection. In the liver, however, sugars would appear to be in plentiful supply, as numerous genes involved in carbohydrate metabolism (for example, *PFK1*, encoding a key enzyme of glycolysis; *PDA1* and *PDX1*, involved in acetyl-CoA biosynthesis; and *KGD1* and *KGD2*, encoding key enzymes of the tricarboxylic acid cycle) are upregulated (98). Such a scenario fits well with known aspects of the mammalian liver; for example, the liver performs numerous steps in carbohydrate metabolism, including gluconeogenesis and glycogenolysis (the generation of glucose via glycogen catabolism). Therefore, it is possible that C. *albicans* can access glucose and/or other sugars during liver invasion, and the transcriptional profiling of Thewes et al. (98) supports this. However, at least a subpopulation of cells were probably unable to access a ready supply of sugars, as *PFK2* (encoding the key enzyme of gluconeogenesis), *MAL2*, and *ACO1* were also upregulated, especially at later time points. It is possible that these reflect C. *albicans* cells phagocytosed by or exposed to phagocytes.

Nitrogen Acquisition

The liver is also the major site of nitrogen metabolism in the human body and probably a rich source of amino acids and other nitrogen sources, as it plays a major role in the biosynthesis of nonessential amino acids and is also the target organ for alanine-mediated recycling of nitrogen from peripheral sites such as muscle tissue. Again, the transcriptome of C. *albicans* cells invading liver tissue reflected the rich nitrogen state of this organ, as genes associated with nitrogen starvation were not upregulated. Moreover, a large number of genes involved in amino acid metabolism were downregulated, further supporting the view that C. *albicans* has sufficient access to host amino acids in liver tissue. However, although amino acids appeared to be in plentiful supply, certain members of the *SAP* gene family were transcriptionally upregulated.

SAP5 and *-6*, which have both been described as hypha-associated genes, were strongly induced during all stages of liver invasion. This stands to reason, as the majority of cells were growing in the hyphal form. Expression of *SAP2* did not change considerably during the first 3 h of infection; however, at 5 h postinfection, *SAP2* transcript levels were upregulated ~70-fold. Similar results have been found by Staib et al. (93) using in vivo expression technology during intraperitoneal infection. From extensive in vitro studies, *SAP2* (the major protease involved in utilization of proteins as a nitrogen source and predominantly induced in yeast cells) was thought to be specifically expressed when protein is the sole nitrogen source and repressed by the presence of amino acids. However, the expression profile of other nitrogen assimilation genes suggested that other, more favorable nitrogen sources (including amino acids) were available. So do C. *albicans* cells, at 5 h postinfection, run out of free nitrogen and have to begin protein degradation in order to release available nitrogen? Possibly; however, the induction of *SAP2* at later stages of liver invasion may reflect a more general pathogenicity mechanism: the broad degradation of host components, which would release nutrients and may assist further invasion or the establishment of microcolonies, which may consist of a mixture of yeast as well as hyphal cells. Perhaps it is also no coincidence that *SAP2* induction is delayed until 5 h postinfection. In vitro studies have convincingly demonstrated that although the Sap2 enzyme has the broadest substrate specificity of the Sap family, its pH optimum is around 4 (58)—far lower than the pH of the intraperitoneal cavity, or indeed, native liver tissue itself. Indeed, a healthy liver has a neutral/mildly alkaline pH, so why would C. *albicans* upregulate expression of a nonfunctional enzyme ~70-fold? As mentioned in above sections, C. *albicans* may be able to actively acidify its local environment, for example, when cells begin to establish microcolonies (microabscesses), which may consist of a mixture of cell morphologies. Such an event is supported by the transcriptional profile of C. *albicans* during the time course of liver invasion. The neutral/alkaline pH marker gene, *PHR1*, is upregulated upon intraperitoneal infection (30 min), reflecting a shift to the neutral/alkaline pH of the intraperitoneal cavity, and expression continues to rise during capsule invasion (3 h) but begins to decrease by 5 h. Expression of the acidic pH marker gene, *PHR2*, is 2-fold higher in the inoculum (0 min; overnight YPD culture) than in the common reference (mid-log-phase YPD), in line with C. *albicans* acidifying the surrounding (YPD) medium following overnight growth. Upon infection, *PHR2* expression falls 5-fold, reflecting a rapid adaptation to the neutral/alkaline environment of the intraperitoneal cavity. However, at later time points, *PHR2* expression rises again and by 5 h postinfection has reached levels higher than in the inoculum. The expression profile of these two pH marker genes suggests that although C. *albicans* encounters a neutral/alkaline pH in the intraperitoneal cavity, on the capsule of the liver and during initial invasion of liver, at least a subpopulation of cells is growing within a local acidic environment by later time points. It is possible that such fluctuations in pH are due to subpopulations of cells being exposed to the acidic environment of phagolysosomes. Alternatively, C. *albicans* may be actively acidifying its local environment. At the later time points of liver invasion (3 and 5 h), *PMA1*, which encodes the major plasma membrane H^+-ATPase, is strongly (>7-fold) induced in comparison to early time points (0 and 30 min). C*aPMA1* is a functional homologue of its *S. cerevisiae* counterpart (54), and in brewer's yeast, *PMA1* is an essential housekeeping gene, the product of which is an abundant plasma membrane protein which actively pumps protons out of the cell. Although the function of Pma1 in *S. cerevisiae* is the generation of a proton gradient to regulate cytoplasmic pH and drive the secondary import of nutrients across the plasma membrane, it is certainly possible that C. *albicans* has adapted its function to acidify its surrounding host environment, thus activating Sap2 proteolytic activity as well as promoting other forms of nutrient uptake such as iron acquisition (see below).

Iron Acquisition

Despite the apparent abundance of carbon and nitrogen sources, iron limitation would appear to represent an important challenge for *C. albicans* in the liver. This is unsurprising, as mammalian hosts tightly restrict microbial access to iron in a process known as nutritional immunity (3). This severe iron limitation was reflected in the transcriptome of *C. albicans*: numerous genes associated with high-affinity iron uptake (which is mediated by the reductive pathway in *C. albicans*) were upregulated during liver invasion. These included the gene encoding the high-affinity iron permease (*FTR1*), two ferric reductase genes (*FRE5*/*FRP1* and *CFL1*), a multicopper ferroxidase gene (*FET5*/*FET34*), and a high-affinity copper transporter gene (*CTR1*) (note: copper import is essential for high-affinity iron uptake because the ferroxidases of the reductive pathway rely on intracellular copper to function).

Although free iron is highly restricted (effectively absent) in fluids and extracellular spaces of the host, *C. albicans* has developed mechanisms to exploit host iron-binding proteins, and iron sequestration from a number of host proteins has been described (3). The liver is the major site of transferrin synthesis in humans, and *C. albicans* has been demonstrated to exploit transferrin as an iron source (43); interestingly, iron acquisition from transferrin is mediated by the reductive pathway—deletion of *FTR1* in *C. albicans* renders the fungus unable to utilize transferrin iron. Therefore, coordinated upregulation of genes of the reductive pathway may permit transferrin iron utilization during liver infection. However, although *C. albicans* requires contact with transferrin to acquire its iron load, a transferrin receptor has yet to be identified.

C. albicans can also acquire iron from host hemoglobin, and for two proteins (Rbt5 and Rbt51/Pga10), hemoglobin-binding properties have been demonstrated. Moreover, *RBT5* and *RBT51*/*PGA10* belong to a gene family (which also contains the predicted hemoglobin receptor genes *WAP1*/*CSA1*, *CSA2*, and *PGA7*). All five members of the hemoglobin receptor gene family are strongly upregulated during the invasive/late stages of liver invasion, and interestingly, the expression patterns of all five genes are virtually identical. It is therefore tempting to speculate that invading fungal cells utilize hemoglobin-derived iron. However, the heme oxygenase (encoded by *HMX1*) was transcriptionally downregulated in the liver. *HMX1* has no other homologue in the *C. albicans* genome, is transcriptionally induced in vitro in response to hemin, and has been demonstrated to be essential in vitro for utilization of iron from hemoglobin (70). It is possible that the low level of *HMX1* expression in the liver is sufficient to release iron from hemoglobin; alternatively, expression of the hemoglobin receptor arsenal by *C. albicans* may represent a distinct pathogenicity mechanism. For example, it has been shown that hemoglobin induces *C. albicans* binding to host extracellular matrix proteins (such as fibronectin, laminin, fibrinogen, and type IV collagen) (71) in an iron acquisition-independent manner.

C. albicans can also utilize iron from the major intracellular iron storage protein ferritin, and in fact, along with the spleen and bone marrow, the liver contains the highest concentration of ferritin in the human body. Like transferrin, ferritin iron exploitation by *C. albicans* also relies on the reductive pathway, so the upregulation of components of this pathway would certainly make ferritin iron exploitation possible. Moreover, the *C. albicans* adhesin and invasin encoded by *ALS3* also serves as a ferritin receptor (2), and *ALS3* is strongly upregulated during liver infection. Another iron acquisition obstacle faced by the fungus is the relatively alkaline pH of the liver (pH 7.4), which favors the insoluble (Fe^{3+}) form of iron. However, as detailed above, it is possible that *C. albicans* is able to actively acidify its local environment and hence shift iron to its ferrous state. Indeed, the process of local environmental acidification has been proposed to be an essential stage in liberation of iron load from both transferrin and ferritin (2, 3). Transcriptional profiling of liver infection suggests that *C. albicans* most likely faces an iron-poor environment but overcomes this by taking advantage of multiple host iron-binding proteins present in the liver. It is also likely that *C. albicans* faces a phosphate-poor environment during liver invasion, as indicated by upregulation of the gene encoding the high-affinity phosphate transporter *PHO84*.

Stress Response

Compared to other body sites, the general, environmental stresses faced by *C. albicans* also appeared to be different in the liver. For example, unlike in oral tissue, *C. albicans* did not appear to face significant nitrosative stress during liver infection, as nitrosative stress marker genes (*YHB5*, *SSU1*, and *YHB1*) were not upregulated during invasion of this organ. Interestingly, numerous genes involved in response to thermal stress—*HSP78*, *HSP90*, *HSP104*, *HSP12*, and *HSP70*—were induced during liver invasion. The induction of the corresponding heat shock proteins may be in response to a fever-induced elevation in temperature upon intraperitoneal infection; however, even artificial fever induction by intraperitoneal injection of the proinflammatory cytokine tumor necrosis factor alpha has been shown to increase mouse body temperature by only 1°C (96), and it is unclear whether this would be a sufficient increase in temperature for *C. albicans* to mount a heat shock response. Indeed, of these five heat shock protein-encoding genes, only *HSP70* and *HSP104* are substantially (>2-fold) induced upon a temperature shift from 23 to 37°C, while the others require a shift to higher temperatures (42 or 45°C) for transcriptional upregulation (63). Interestingly, while all five of these heat shock proteins were strongly induced during in vivo mouse infection, none of them were transcriptionally upregulated upon ex vivo pig liver infection, so dynamic temperature fluctuations in a living host may still provide an explanation for heat shock protein induction.

Another possibility is that the upregulated chaperones are induced by environmental conditions other than thermal stress. For example, *HSP78* has been shown to be strongly induced following engulfment by macrophages (49); *HSP12* is induced upon iron starvation, which fits with the general idea of iron limitation during liver invasion (above). Finally, *HSP70* plays a role in resistance to β-defensins (104); although β-defensins are typically found at mucosal surfaces, their expression in rat liver has been reported (29) and may therefore represent a genuine threat to *C. albicans* during liver invasion. Moreover, Hsp70 (as well as other members of the Hsp70 family) has been shown to be highly immunogenic in mice (13, 73), which may also promote a proinflammatory-cytokine-mediated fever.

There was not a clear oxidative stress response in the liver, although *SOD5*/*PGA3*, which encodes a major superoxide dismutase and plays a central role in oxidative stress tolerance (28, 51), was over 50-fold upregulated during liver invasion. This may be due to hyphal growth, rather than substantial environmental oxidative stress caused by infiltrating

phagocytes: *SOD5* is, in the most part, a hypha-coregulated gene, and its induction is not strictly dependent on exogenous reactive oxygen species (28, 51). Indeed coupling *SOD5* upregulation to (invasive) hyphal formation, irrespective of exogenous oxygen radical levels, may represent an effective pathogenicity or immune defense strategy for *C. albicans*. Even if substantial oxidative stress is a relatively rare occurrence during tissue invasion (as has been indicated by single-cell profiling during infection of mouse kidneys; see above), it may be a sufficiently adverse environmental insult for it to be beneficial for the fungus to prepare in advance by upregulating *SOD5* in an oxidative stress-independent manner. This concept of "adaptive prediction" (53), which is analogous to Pavlovian conditioning, has been described for other organisms. The general stress response of *S. cerevisiae*, where preexposure to one environmental insult protects against other stresses, has been well described (47, 77). However, a more sophisticated system, known as adaptive prediction or environmental or predictive adaptation, has recently been described for both *Escherichia coli* and *Saccharomyces cerevisiae* and may represent a universal biological strategy for dealing with changing environments (53).

OUTLOOK AND FUTURE PERSPECTIVES

In summary, although transcriptional profiling of *C. albicans* in vivo is still in its infancy, the available data sets already provide an exciting insight into the real in vivo situation. For example, the upregulation of distinct gene sets in particular organs has provided clues of the environmental conditions faced by the fungus in these niches.

However, to take full advantage of both existing and forthcoming in vivo data sets, a more detailed insight into the nature of this enigmatic fungus and its interaction with host cells and complex host environments will be required.

First, interpretation of complex in vivo microarray studies is hampered by an incomplete understanding of gene expression under more simple in vitro conditions. For example, the expression of particular marker genes in *C. albicans* is not always strictly limited to the environmental condition in question. Moreover, many proteins probably have multiple functions and/or are involved in multiple cellular processes. Taken together, all this means that the upregulation of a particular gene during an infection is not conclusive evidence of a particular environmental stimulus occurring within that niche. Rather, we must look for groups of genes (regulons) associated with particular environmental conditions. Although a number of studies have been performed, providing insight into the transcriptional response of *C. albicans* in response to changing pH, different environmental stresses, drug treatment, the yeast-to-hypha transition, and limited iron conditions (10, 23, 38, 45, 48, 62–64), these should be extended to cover a more comprehensive range of defined environmental conditions and to identify regulons associated with particular conditions. As the number of published microarray studies expands, systems biology will play an increasingly important role, both in the interpretation of large-scale data sets and in developing hypotheses and designing future experiments.

Second, for a large proportion of *C. albicans* genes, the molecular function remains poorly defined, and relying on (sometimes limited) sequence similarity in *S. cerevisiae* is not always satisfactory, further complicating the interpretation of genome-wide transcriptional analyses. Therefore, a second challenge will be to extend our functional coverage of the *C. albicans* genome. Determining the function of uncharacterized genes, particularly of those not present in other nonpathogenic yeast species and expressed during interaction with host cells (Wilson et al., unpublished), will provide greater insight into the molecular mechanisms underlying the pathogenicity of candidiasis.

Furthermore, the next decade will surely witness a significant expansion in the number of in vivo transcriptional profiling studies performed, covering a wider range of infection sites in greater temporal and spatial detail. This expansion, combined with advancements in the functional characterization of the *C. albicans* genome and systems biology approaches to integrate the resultant wealth of data, will drive forward our understanding of the molecular mechanisms underpinning candidiasis.

REFERENCES

1. **Alberti-Segui, C., A. J. Morales, H. Xing, M. M. Kessler, D. A. Willins, K. G. Weinstock, G. Cottarel, K. Fechtel, and B. Rogers.** 2004. Identification of potential cell-surface proteins in *Candida albicans* and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence. *Yeast* (Chichester) **21:**285–302.
2. **Almeida, R. S., S. Brunke, A. Albrecht, S. Thewes, M. Laue, J. E. Edwards, S. G. Filler, and B. Hube.** 2008. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog.* **4:**e1000217.
3. **Almeida, R. S., D. Wilson, and B. Hube.** 2009. Candida albicans iron acquisition within the host. *FEMS Yeast Res.* **9:**1000–1012.
4. **Andes, D., A. Lepak, A. Pitula, K. Marchillo, and J. Clark.** 2005. A simple approach for estimating gene expression in *Candida albicans* directly from a systemic infection site. *J. Infect. Dis.* **192:**893–900.
5. **Argimon, S., J. A. Wishart, R. Leng, S. Macaskill, A. Mavor, T. Alexandris, S. Nicholls, A. W. Knight, B. Enjalbert, R. Walmsley, F. C. Odds, N. A. Gow, and A. J. Brown.** 2007. Developmental regulation of an adhesin gene during cellular morphogenesis in the fungal pathogen *Candida albicans*. *Eukaryot. Cell* **6:**682–692.
6. **Bailey, D. A., P. J. Feldmann, M. Bovey, N. A. Gow, and A. J. Brown.** 1996. The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. *J. Bacteriol.* **178:**5353–5360.
7. **Barelle, C. J., C. L. Priest, D. M. Maccallum, N. A. Gow, F. C. Odds, and A. J. Brown.** 2006. Niche-specific regulation of central metabolic pathways in a fungal pathogen. *Cell. Microbiol.* **8:**961–971.
8. **Bendel, C. M., S. M. Wiesner, R. M. Garni, E. Cebelinski, and C. L. Wells.** 2002. Cecal colonization and systemic spread of *Candida albicans* in mice treated with antibiotics and dexamethasone. *Pediatr. Res.* **51:**290–295.
9. **Bensen, E. S., S. G. Filler, and J. Berman.** 2002. A forkhead transcription factor is important for true hyphal as well as yeast morphogenesis in *Candida albicans*. *Eukaryot. Cell* **1:**787–798.
10. **Bensen, E. S., S. J. Martin, M. Li, J. Berman, and D. A. Davis.** 2004. Transcriptional profiling in *Candida albicans* reveals new adaptive responses to extracellular pH and functions for Rim101p. *Mol. Microbiol.* **54:**1335–1351.
11. **Birse, C. E., M. Y. Irwin, W. A. Fonzi, and P. S. Sypherd.** 1993. Cloning and characterization of *ECE1*, a gene expressed in association with cell elongation of the dimorphic pathogen *Candida albicans*. *Infect. Immun.* **61:**3648–3655.

12. **Braun, B. R., W. S. Head, M. X. Wang, and A. D. Johnson.** 2000. Identification and characterization of TUP1-regulated genes in *Candida albicans*. *Genetics* **156:**31–44.
13. **Bromuro, C., R. La Valle, S. Sandini, F. Urbani, C. M. Ausiello, L. Morelli, C. Fe d'Ostiani, L. Romani, and A. Cassone.** 1998. A 70-kilodalton recombinant heat shock protein of *Candida albicans* is highly immunogenic and enhances systemic murine candidiasis. *Infect. Immun.* **66:** 2154–2162.
14. **Brown, J. F., and E. Balish.** 1978. Gastrointestinal microecology of BALB/c nude mice. *Appl. Environ. Microbiol.* **36:**144–159.
15. **Cole, G. T., K. T. Lynn, K. R. Seshan, and L. M. Pope.** 1989. Gastrointestinal and systemic candidosis in immunocompromised mice. *J. Med. Vet. Mycol.* **27:**363–380.
16. **Dalle, F., B. Wachtler, C. L'Ollivier, G. Holland, N. Bannert, D. Wilson, C. Labruere, A. Bonnin, and B. Hube.** Cellular interactions of *Candida albicans* with human oral epithelial cells and enterocytes. *Cell. Microbiol.* **12:**248–271.
17. **De Bernardis, F., S. Arancia, L. Morelli, B. Hube, D. Sanglard, W. Schafer, and A. Cassone.** 1999. Evidence that members of the secretory aspartyl proteinase gene family, in particular SAP2, are virulence factors for *Candida* vaginitis. *J. Infect. Dis.* **179:**201–208.
18. **De Bernardis, F., F. A. Muhlschlegel, A. Cassone, and W. A. Fonzi.** 1998. The pH of the host niche controls gene expression in and virulence of *Candida albicans*. *Infect. Immun.* **66:**3317–3325.
19. **Doedt, T., S. Krishnamurthy, D. P. Bockmuhl, B. Tebarth, C. Stempel, C. L. Russell, A. J. Brown, and J. F. Ernst.** 2004. APSES proteins regulate morphogenesis and metabolism in *Candida albicans*. *Mol. Biol. Cell* **15:** 3167–3180.
20. **Dongari-Bagtzoglou, A., and H. Kashleva.** 2006. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. *Nat. Protoc.* **1:**2012–2018.
21. **Ekenna, O., and R. J. Sherertz.** 1987. Factors affecting colonization and dissemination of *Candida albicans* from the gastrointestinal tract of mice. *Infect. Immun.* **55:**1558–1563.
22. **Enjalbert, B., D. M. MacCallum, F. C. Odds, and A. J. Brown.** 2007. Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. *Infect. Immun.* **75:**2143–2151.
23. **Enjalbert, B., D. A. Smith, M. J. Cornell, I. Alam, S. Nicholls, A. J. Brown, and J. Quinn.** 2006. Role of the Hog1 stress-activated protein kinase in the global transcriptional response to stress in the fungal pathogen *Candida albicans*. *Mol. Biol. Cell* **17:**1018–1032.
24. **Ferrer, J.** 2000. Vaginal candidosis: epidemiological and etiological factors. *Int. J. Gynaecol. Obstet.* **71**(Suppl. 1)**:** S21–S27.
25. **Fidel, P. L., Jr., and J. D. Sobel.** 1996. Immunopathogenesis of recurrent vulvovaginal candidiasis. *Clin. Microbiol. Rev.* **9:**335–348.
26. **Filler, S. G., and D. C. Sheppard.** 2006. Fungal invasion of normally non-phagocytic host cells. *PLoS Pathog.* **2:**e129.
27. **Fonzi, W. A.** 1999. *PHR1* and *PHR2* of *Candida albicans* encode putative glycosidases required for proper cross-linking of β-1,3- and β-1,6-glucans. *J. Bacteriol.* **181:**7070–7079.
28. **Fradin, C., P. De Groot, D. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube.** 2005. Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol. Microbiol.* **56:**397–415.
29. **Froy, O., A. Hananel, N. Chapnik, and Z. Madar.** 2005. Differential expression of rat beta-defensins. *IUBMB Life* **57:**41–43.
30. **Fu, Y., A. S. Ibrahim, D. C. Sheppard, Y. C. Chen, S. W. French, J. E. Cutler, S. G. Filler, and J. E. Edwards, Jr.** 2002. *Candida albicans* Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. *Mol. Microbiol.* **44:**61–72.
31. **Garcia-Sanchez, S., S. Aubert, I. Iraqui, G. Janbon, J. M. Ghigo, and C. d'Enfert.** 2004. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot. Cell* **3:**536–545.
32. **Giusani, A. D., M. Vinces, and C. A. Kumamoto.** 2002. Invasive filamentous growth of *Candida albicans* is promoted by Czf1p-dependent relief of Efg1p-mediated repression. *Genetics* **160:**1749–1753.
33. **Green, C. B., S. M. Marretta, G. Cheng, F. F. Faddoul, E. J. Ehrhart, and L. L. Hoyer.** 2006. RT-PCR analysis of *Candida albicans* ALS gene expression in a hyposalivatory rat model of oral candidiasis and in HIV-positive human patients. *Med. Mycol.* **44:**103–111.
34. **Grubb, S. E., C. Murdoch, P. E. Sudbery, S. P. Saville, J. L. Lopez-Ribot, and M. H. Thornhill.** 2009. Adhesion of *Candida albicans* to endothelial cells under physiological conditions of flow. *Infect. Immun.* **77:**3872–3878.
35. **Guentzel, M. N., and C. Herrera.** 1982. Effects of compromising agents on candidosis in mice with persistent infections initiated in infancy. *Infect. Immun.* **35:**222–228.
36. **Hoyer, L. L., C. B. Green, S. H. Oh, and X. Zhao.** 2008. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family—a sticky pursuit. *Med. Mycol.* **46:**1–15.
37. **Hoyer, L. L., T. L. Payne, M. Bell, A. M. Myers, and S. Scherer.** 1998. *Candida albicans* ALS3 and insights into the nature of the ALS gene family. *Curr. Genet.* **33:**451–459.
38. **Hromatka, B. S., S. M. Noble, and A. D. Johnson.** 2005. Transcriptional response of *Candida albicans* to nitric oxide and the role of the *YHB1* gene in nitrosative stress and virulence. *Mol. Biol. Cell* **16:**4814–4826.
39. **Hube, B.** 2004. From commensal to pathogen: stage- and tissue-specific gene expression of *Candida albicans*. *Curr. Opin. Microbiol.* **7:**336–341.
40. **Hube, B.** 2009. Fungal adaptation to the host environment. *Curr. Opin. Microbiol.* **12:**347–349.
41. **Hube, B., and J. Naglik.** 2001. Candida albicans proteinases: resolving the mystery of a gene family. *Microbiology* (Reading) **147:**1997–2005.
42. **Jong, A. Y., M. F. Stins, S. H. Huang, S. H. Chen, and K. S. Kim.** 2001. Traversal of *Candida albicans* across human blood-brain barrier in vitro. *Infect. Immun.* **69:**4536–4544.
43. **Knight, S. A., G. Vilaire, E. Lesuisse, and A. Dancis.** 2005. Iron acquisition from transferrin by *Candida albicans* depends on the reductive pathway. *Infect. Immun.* **73:** 5482–5492.
44. **Koh, A. Y., J. R. Kohler, K. T. Coggshall, N. Van Rooijen, and G. B. Pier.** 2008. Mucosal damage and neutropenia are required for *Candida albicans* dissemination. *PLoS Pathog.* **4:**e35.
45. **Lan, C. Y., G. Rodarte, L. A. Murillo, T. Jones, R. W. Davis, J. Dungan, G. Newport, and N. Agabian.** 2004. Regulatory networks affected by iron availability in *Candida albicans*. *Mol. Microbiol.* **53:**1451–1469.
46. **Lee, R. E., T. T. Liu, K. S. Barker, R. E. Lee, and P. D. Rogers.** 2005. Genome-wide expression profiling of the response to ciclopirox olamine in *Candida albicans*. *J. Antimicrob. Chemother.* **55:**655–662.
47. **Lewis, J. G., R. P. Learmonth, and K. Watson.** 1995. Induction of heat, freezing and salt tolerance by heat and salt shock in *Saccharomyces cerevisiae*. *Microbiology* (Reading) **141**(Pt. 3)**:**687–694.
48. **Liu, T. T., R. E. Lee, K. S. Barker, R. E. Lee, L. Wei, R. Homayouni, and P. D. Rogers.** 2005. Genome-wide

expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob. Agents Chemother.* **49:**2226–2236.
49. **Lorenz, M. C., J. A. Bender, and G. R. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot. Cell* **3:**1076–1087.
50. **MacCallum, D. M., and F. C. Odds.** 2005. Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. *Mycoses* **48:**151–161.
51. **Martchenko, M., A. M. Alarco, D. Harcus, and M. Whiteway.** 2004. Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced SOD5 gene. *Mol. Biol. Cell* **15:**456–467.
52. **Mavor, A. L., S. Thewes, and B. Hube.** 2005. Systemic fungal infections caused by *Candida* species: epidemiology, infection process and virulence attributes. *Curr. Drug Targets* **6:**863–874.
53. **Mitchell, A., G. H. Romano, B. Groisman, A. Yona, E. Dekel, M. Kupiec, O. Dahan, and Y. Pilpel.** 2009. Adaptive prediction of environmental changes by microorganisms. *Nature* **460:**220–224.
54. **Monk, B. C., M. B. Kurtz, J. A. Marrinan, and D. S. Perlin.** 1991. Cloning and characterization of the plasma membrane H^+-ATPase from *Candida albicans*. *J. Bacteriol.* **173:**6826–6836.
55. **Monteoliva, L., M. Sanchez, J. Pla, C. Gil, and C. Nombela.** 1996. Cloning of *Candida albicans* SEC14 gene homologue coding for a putative essential function. *Yeast* (Chichester) **12:**1097–1105.
56. **Muhlschlegel, F. A., and W. A. Fonzi.** 1997. *PHR2* of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. *Mol. Cell. Biol.* **17:**5960–5967.
57. **Naglik, J., A. Albrecht, O. Bader, and B. Hube.** 2004. *Candida albicans* proteinases and host/pathogen interactions. *Cell. Microbiol.* **6:**915–926.
58. **Naglik, J. R., S. J. Challacombe, and B. Hube.** 2003. *Candida albicans* secreted aspartyl proteinases in virulence and pathogenesis. *Microbiol. Mol. Biol. Rev.* **67:**400–428.
59. **Naglik, J. R., P. L. Fidel, Jr., and F. C. Odds.** 2008. Animal models of mucosal *Candida* infection. *FEMS Microbiol. Lett.* **283:**129–139.
60. **Naglik, J. R., F. Fostira, J. Ruprai, J. F. Staab, S. J. Challacombe, and P. Sundstrom.** 2006. *Candida albicans* HWP1 gene expression and host antibody responses in colonization and disease. *J. Med. Microbiol.* **55:**1323–1327.
61. **Nakagawa, Y., K. Koide, K. Watanabe, Y. Morita, I. Mizuguchi, and T. Akashi.** 1999. The expression of the pathogenic yeast *Candida albicans* catalase gene in response to hydrogen peroxide. *Microbiol. Immunol.* **43:**645–651.
62. **Nantel, A., D. Dignard, C. Bachewich, D. Harcus, A. Marcil, A. P. Bouin, C. W. Sensen, H. Hogues, M. van het Hoog, P. Gordon, T. Rigby, F. Benoit, D. C. Tessier, D. Y. Thomas, and M. Whiteway.** 2002. Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol. Biol. Cell* **13:**3452–3465.
63. **Nicholls, S., M. D. Leach, C. L. Priest, and A. J. Brown.** 2009. Role of the heat shock transcription factor, Hsf1, in a major fungal pathogen that is obligately associated with warm-blooded animals. *Mol. Microbiol.* **74:**844–861.
64. **Niewerth, M., D. Kunze, M. Seibold, M. Schaller, H. C. Korting, and B. Hube.** 2003. Ciclopirox olamine treatment affects the expression pattern of *Candida albicans* genes encoding virulence factors, iron metabolism proteins, and drug resistance factors. *Antimicrob. Agents Chemother.* **47:**1805–1817.
65. **Nucci, M., and E. Anaissie.** 2001. Revisiting the source of candidemia: skin or gut? *Clin. Infect. Dis.* **33:**1959–1967.
66. **Ostrander, D. B., and J. A. Gorman.** 1994. Characterization of the *Candida albicans TRP1* gene and construction of a homozygous trp1 mutant by sequential co-transformation. *Gene* **148:**179–185.
67. **Pappas, P. G., J. H. Rex, J. D. Sobel, S. G. Filler, W. E. Dismukes, T. J. Walsh, and J. E. Edwards.** 2004. Guidelines for treatment of candidiasis. *Clin. Infect. Dis.* **38:**161–189.
68. **Park, H., Y. Liu, N. Solis, J. Spotkov, J. Hamaker, J. R. Blankenship, M. R. Yeaman, A. P. Mitchell, H. Liu, and S. G. Filler.** 2009. Transcriptional responses of *Candida albicans* to epithelial and endothelial cells. *Eukaryot. Cell* **8:**1498–1510.
69. **Paulitsch, A., W. Weger, G. Ginter-Hanselmayer, E. Marth, and W. Buzina.** 2006. A 5-year (2000–2004) epidemiological survey of *Candida* and non-*Candida* yeast species causing vulvovaginal candidiasis in Graz, Austria. *Mycoses* **49:**471–475.
70. **Pendrak, M. L., M. P. Chao, S. S. Yan, and D. D. Roberts.** 2004. Heme oxygenase in *Candida albicans* is regulated by hemoglobin and is necessary for metabolism of exogenous heme and hemoglobin to alpha-biliverdin. *J. Biol. Chem.* **279:**3426–3433.
71. **Pendrak, M. L., S. S. Yan, and D. D. Roberts.** 2004. Sensing the host environment: recognition of hemoglobin by the pathogenic yeast *Candida albicans*. *Arch. Biochem. Biophys.* **426:**148–156.
72. **Phan, Q. T., C. L. Myers, Y. Fu, D. C. Sheppard, M. R. Yeaman, W. H. Welch, A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol.* **5:**e64.
73. **Pitarch, A., R. Diez-Orejas, G. Molero, M. Pardo, M. Sanchez, C. Gil, and C. Nombela.** 2001. Analysis of the serologic response to systemic *Candida albicans* infection in a murine model. *Proteomics* **1:**550–559.
74. **Pultz, N. J., U. Stiefel, M. Ghannoum, M. S. Helfand, and C. J. Donskey.** 2005. Effect of parenteral antibiotic administration on establishment of intestinal colonization by *Candida glabrata* in adult mice. *Antimicrob. Agents Chemother.* **49:**438–440.
75. **Ramon, A. M., and W. A. Fonzi.** 2003. Diverged binding specificity of Rim101p, the *Candida albicans* ortholog of PacC. *Eukaryot. Cell* **2:**718–728.
76. **Rubin-Bejerano, I., I. Fraser, P. Grisafi, and G. R. Fink.** 2003. Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **100:**11007–11012.
76a. **Ruhnke, M.** 2002. Skin and mucous membrane infections, p. 307–325. *In* R. Calderone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
77. **Ruis, H., and C. Schuller.** 1995. Stress signaling in yeast. *Bioessays* **17:**959–965.
78. **Samonis, G., E. Mantadakis, E. Barbounakis, D. Kofteridis, G. Papadakis, L. Sifaki, and S. Maraki.** 2008. Effects of tigecycline and daptomycin on murine gut colonization by *Candida albicans*. *Mycoses* **51:**324–327.
79. **Sandovsky-Losica, H., L. Barr-Nea, and E. Segal.** 1992. Fatal systemic candidiasis of gastrointestinal origin: an experimental model in mice compromised by anti-cancer treatment. *J. Med. Vet. Mycol.* **30:**219–231.
80. **Sandovsky-Losica, H., N. Chauhan, R. Calderone, and E. Segal.** 2006. Gene transcription studies of *Candida albicans* following infection of HEp2 epithelial cells. *Med. Mycol.* **44:**329–334.

81. **Saporito-Irwin, S. M., C. E. Birse, P. S. Sypherd, and W. A. Fonzi.** 1995. PHR1, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. *Mol. Cell. Biol.* **15:**601–613.
82. **Saville, S. P., A. L. Lazzell, A. P. Bryant, A. Fretzen, A. Monreal, E. O. Solberg, C. Monteagudo, J. L. Lopez-Ribot, and G. T. Milne.** 2006. Inhibition of filamentation can be used to treat disseminated candidiasis. *Antimicrob. Agents Chemother.* **50:**3312–3316.
83. **Saville, S. P., A. L. Lazzell, C. Monteagudo, and J. L. Lopez-Ribot.** 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot. Cell* **2:**1053–1060.
84. **Sawyer, R. T., R. J. Moon, and E. S. Beneke.** 1976. Hepatic clearance of *Candida albicans* in rats. *Infect. Immun.* **14:**1348–1355.
85. **Schaller, M., W. Schafer, H. C. Korting, and B. Hube.** 1998. Differential expression of secreted aspartyl proteinases in a model of human oral candidosis and in patient samples from the oral cavity. *Mol. Microbiol.* **29:** 605–615.
86. **Schaller, M., K. Zakikhany, J. R. Naglik, G. Weindl, and B. Hube.** 2006. Models of oral and vaginal candidiasis based on in vitro reconstituted human epithelia. *Nat. Protoc.* **1:**2767–2773.
87. **Schofield, D. A., C. Westwater, T. Warner, and E. Balish.** 2005. Differential *Candida albicans* lipase gene expression during alimentary tract colonization and infection. *FEMS Microbiol. Lett.* **244:**359–365.
88. **Schroter, C., U. C. Hipler, A. Wilmer, W. Kunkel, and U. Wollina.** 2000. Generation of reactive oxygen species by *Candida albicans* in relation to morphogenesis. *Arch. Dermatol. Res.* **292:**260–264.
89. **Setiadi, E. R., T. Doedt, F. Cottier, C. Noffz, and J. F. Ernst.** 2006. Transcriptional response of *Candida albicans* to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. *J. Mol. Biol.* **361:**399–411.
90. **Sohn, K., I. Senyurek, J. Fertey, A. Konigsdorfer, C. Joffroy, N. Hauser, G. Zelt, H. Brunner, and S. Rupp.** 2006. An in vitro assay to study the transcriptional response during adherence of *Candida albicans* to different human epithelia. *FEMS Yeast Res.* **6:**1085–1093.
91. **Sosinska, G. J., P. W. de Groot, M. J. Teixeira de Mattos, H. L. Dekker, C. G. de Koster, K. J. Hellingwerf, and F. M. Klis.** 2008. Hypoxic conditions and iron restriction affect the cell-wall proteome of *Candida albicans* grown under vagina-simulative conditions. *Microbiology* (Reading) **154:** 510–520.
92. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283:** 1535–1538.
93. **Staib, P., M. Kretschmar, T. Nichterlein, G. Kohler, S. Michel, H. Hof, J. Hacker, and J. Morschhauser.** 1999. Host-induced, stage-specific virulence gene activation in *Candida albicans* during infection. *Mol. Microbiol.* **32:** 533–546.
94. **Stichternoth, C., and J. F. Ernst.** 2009. Hypoxic adaptation by Efg1 regulates biofilm formation by *Candida albicans*. *Appl. Environ. Microbiol.* **75:**3663–3672.
95. **Stribinskis, V., H. C. Heyman, S. R. Ellis, M. C. Steffen, and N. C. Martin.** 2005. Rpm2p, a component of yeast mitochondrial RNase P, acts as a transcriptional activator in the nucleus. *Mol. Cell. Biol.* **25:**6546–6558.
96. **Sundgren-Andersson, A. K., P. Ostlund, and T. Bartfai.** 1998. IL-6 is essential in TNF-alpha-induced fever. *Am. J. Physiol.* **275:**R2028–R2034.
97. **Taylor, B. N., P. Staib, A. Binder, A. Biesemeier, M. Sehnal, M. Rollinghoff, J. Morschhauser, and K. Schroppel.** 2005. Profile of *Candida albicans*-secreted aspartic proteinase elicited during vaginal infection. *Infect. Immun.* **73:**1828–1835.
98. **Thewes, S., M. Kretschmar, H. Park, M. Schaller, S. G. Filler, and B. Hube.** 2007. In vivo and ex vivo comparative transcriptional profiling of invasive and non-invasive *Candida albicans* isolates identifies genes associated with tissue invasion. *Mol. Microbiol.* **63:** 1606–1628.
99. **Timpel, C., S. Zink, S. Strahl-Bolsinger, K. Schroppel, and J. Ernst.** 2000. Morphogenesis, adhesive properties, and antifungal resistance depend on the Pmt6 protein mannosyltransferase in the fungal pathogen *Candida albicans*. *J. Bacteriol.* **182:**3063–3071.
100. **Turner, J. R., T. F. Butler, M. E. Johnson, and R. S. Gordee.** 1976. Colonization of the intestinal tract of conventional mice with *Candida albicans* and treatment with antifungal agents. *Antimicrob. Agents Chemother.* **9:**787–792.
101. **van Ogtrop, M. L., H. F. Guiot, H. Mattie, and R. van Furth.** 1991. Modulation of the intestinal flora of mice by parenteral treatment with broad-spectrum cephalosporins. *Antimicrob. Agents Chemother.* **35:**976–982.
102. **van Ogtrop, M. L., H. Mattie, H. F. Guiot, and R. van Furth.** 1990. Relation between the therapeutic efficacy of imipenem in an experimental infection in granulocytopenic mice and its effect on murine intestinal microbial ecology. *J. Antimicrob. Chemother.* **26:**399–409.
103. **Voss, A., R. J. Hollis, M. A. Pfaller, R. P. Wenzel, and B. N. Doebbeling.** 1994. Investigation of the sequence of colonization and candidemia in nonneutropenic patients. *J. Clin. Microbiol.* **32:**975–980.
104. **Vylkova, S., X. S. Li, J. C. Berner, and M. Edgerton.** 2006. Distinct antifungal mechanisms: beta-defensins require *Candida albicans* Ssa1 protein, while Trk1p mediates activity of cysteine-free cationic peptides. *Antimicrob. Agents Chemother.* **50:**324–331.

104a. **Wächtler, B., D. Wilson, K. Haedicke, F. Dalle, and B. Hube.** 2011. From attachment to damage: defined genes of *Candida albicans* mediate adhesion, invasion and damage during interaction with oral epithelial cells. *PLoS One* **6:**e17046.

105. **Walker, L. A., D. M. Maccallum, G. Bertram, N. A. Gow, F. C. Odds, and A. J. Brown.** 2009. Genome-wide analysis of *Candida albicans* gene expression patterns during infection of the mammalian kidney. *Fungal Genet. Biol.* **46:**210–219.
106. **White, S. J., A. Rosenbach, P. Lephart, D. Nguyen, A. Benjamin, S. Tzipori, M. Whiteway, J. Mecsas, and C. A. Kumamoto.** 2007. Self-regulation of *Candida albicans* population size during GI colonization. *PLoS Pathog.* **3:**e184.
107. **Wilson, D., and B. Hube.** 2010. Hgc1 mediates dynamic *Candida albicans*-endothelium adhesion events during circulation. *Eukaryot. Cell* **9:**278–287.
108. **Wysong, D. R., L. Christin, A. M. Sugar, P. W. Robbins, and R. D. Diamond.** 1998. Cloning and sequencing of a *Candida albicans* catalase gene and effects of disruption of this gene. *Infect. Immun.* **66:**1953–1961.
109. **Zakikhany, K., J. R. Naglik, A. Schmidt-Westhausen, G. Holland, M. Schaller, and B. Hube.** 2007. In vivo transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. *Cell. Microbiol.* **9:**2938–2954.
110. **Zhao, X., S. H. Oh, G. Cheng, C. B. Green, J. A. Nuessen, K. Yeater, R. P. Leng, A. J. Brown, and L. L.**

Hoyer. 2004. *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. *Microbiology* (Reading) **150:**2415–2428.

111. **Zhao, X., S. H. Oh, and L. L. Hoyer.** 2007. Deletion of *ALS5*, *ALS6* or *ALS7* increases adhesion of *Candida albicans* to human vascular endothelial and buccal epithelial cells. *Med. Mycol.* **45:**429–434.
112. **Zhao, X., S. H. Oh, and L. L. Hoyer.** 2007. Unequal contribution of *ALS9* alleles to adhesion between *Candida albicans* and human vascular endothelial cells. *Microbiology* (Reading) **153:**2342–2350.
113. **Zhao, X., S. H. Oh, R. Jajko, D. J. Diekema, M. A. Pfaller, C. Pujol, D. R. Soll, and L. L. Hoyer.** 2007. Analysis of *ALS5* and *ALS6* allelic variability in a geographically diverse collection of *Candida albicans* isolates. *Fungal Genet. Biol.* **44:**1298–1309.
114. **Zhao, X., S. H. Oh, K. M. Yeater, and L. L. Hoyer.** 2005. Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* (Reading) **151:** 1619–1630.
115. **Zhu, W., and S. G. Filler.** Interactions of *Candida albicans* with epithelial cells. *Cell. Microbiol.* **12:**273–282.

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch19

19

Biofilm Formation in *Candida albicans*

JONATHAN SEWELL FINKEL AND AARON P. MITCHELL

This chapter focuses on biofilm formation by the pathogenic fungus *Candida albicans*. Biofilms are surface-associated microbial communities surrounded by an extracellular matrix. *Candida albicans* is a commensal fungus that resides on mucosal surfaces and in the gastrointestinal and genitourinary tracts, where it is benign for most people most of the time. Infection occurs if immune function is impaired or if an environmental niche becomes available (113). Many *C. albicans* infections arise as a result of the organism's ability to grow as a biofilm. Biofilms are clinically significant because of their association with infection due to the presence of an implanted medical device (89). Use of these devices, such as venous catheters, pacemakers, and artificial joints, is routine in the United States, with well over 10 million recipients per year. These devices provide a very substantial health benefit but nonetheless are associated with infection rates as high as 30% (144, 146, 147). These infections are caused or maintained by biofilms that form on device surfaces. Because biofilms are resistant to many antimicrobial agents, treatment often requires removal of infected devices, with later surgery required to replace the infected device. This process is costly and harmful, as increased surgery leads to increased stress and increased frequency of infection in a patient who is already sick (33, 66). The annual cost of antifungal therapies in the United States is estimated at $2.6 billion (148). In this chapter we examine the steps of biofilm formation, from the genes known to function in *C. albicans* biofilm development, the cell-cell communication within the biofilm, the environmental responses that contribute to biofilm formation, the drug resistance of biofilms, and experimental techniques used to study biofilms.

OVERVIEW OF BIOFILM FORMATION

Biofilms are defined as surface-associated microbial communities surrounded by an extracellular matrix. Biofilms are ubiquitous in nature, with nearly every known species of microorganism having mechanisms to adhere to surfaces and to each other. The cells growing in a biofilm are physiologically distinct from planktonic cells or suspended cells by their significantly increased resistance to antimicrobial agents. Biofilms can form on most biological and nonbiological substrates. They are medically important, accounting for ~80% of microbial infections in the body (24). Infections can be found in oral soft tissues; teeth; dental implants; the middle ear; the gastrointestinal tract; the urogenital tract; airway/lung tissue; the eyes; urinary tract prostheses; the peritoneal membrane and peritoneal dialysis catheters; indwelling catheters for hemodialysis and for chronic administration of chemotherapeutic agents (Hickman catheters); cardiac implants such as pacemakers, prosthetic heart valves, ventricular assist devices, and synthetic vascular grafts and stents; prostheses, internal fixation devices, and percutaneous sutures; and tracheal and ventilator tubing (29, 57, 81, 115). While individual organisms can form biofilms, it is not uncommon for multiple organisms to form a mixed biofilm. These mixed biofilms can occur between related species, such as *C. albicans* and *Candida tropicalis*, or between distantly related organisms, such as *C. albicans* and *Staphylococcus epidermidis*, and can confer advantageous resistance to antimicrobial agents (1, 3, 128).

While the majority of biofilm infections are caused by staphylococci, fungal biofilm infections tend to be more detrimental to the host and are an increasing problem (1). While *C. albicans* is the most common cause of candidiasis, other fungal species are being isolated with increasing frequency. *Candida parapsilosis* is now responsible for 8 to 13% of *Candida* bloodstream infections, and *Candida glabrata* is responsible for 24% (47, 118). The initial increase in *C. glabrata* infection rates has been attributed to its reduced azole susceptibility. The incidences of *C. parapsilosis* and *C. glabrata* infections have been associated recently with intrinsic or acquired resistance to antifungal echinocandins due to alterations of the target protein Fks1 (β-1,3 glucan synthase) (36, 38). Infection with *C. parapsilosis* is most common in neonates, in transplant patients, and in patients receiving parenteral nutrition (6, 32, 37, 79, 80). Unlike *C. albicans*, *C. parapsilosis* and *C. glabrata* do not form true hyphae; instead, their biofilms include elongated yeast cells or pseudohyphae (73, 120). Since *C. albicans* is the major pathogen among these, it is the focus of this chapter.

C. albicans can exist as three types of cells: yeast form cells (blastospores), pseudohyphal form cells, and hyphal form cells (Fig. 1). These different cell morphologies allow

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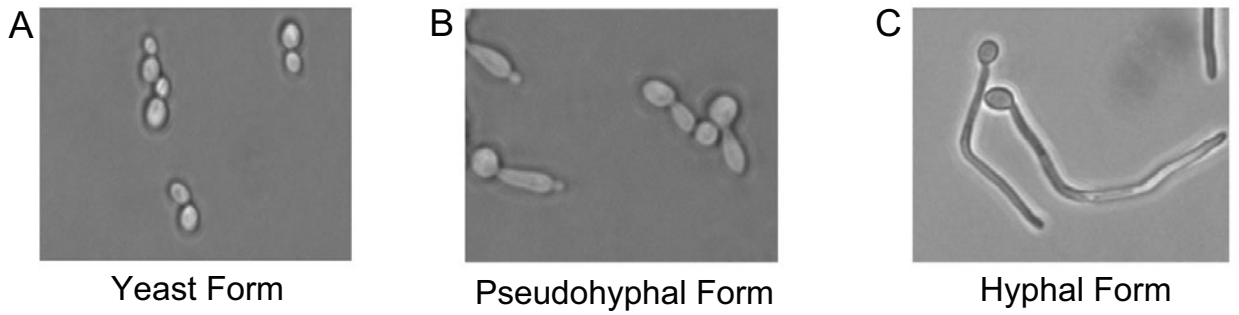

FIGURE 1 The three different morphologies of C. *albicans* cells. (A) Yeast form cells comprise either a single oval cell or an oval mother cell and smaller daughter cells (seen attached to most cells). (B) Pseudohyphal cells, abnormal elongated budding cells, can contain multiple buds. (C) Hyphal cells are large oval cells with long narrow continuous germ tube protrusions. 10.1128/9781555817176.ch19f1

C. *albicans* to rapidly colonize and disseminate in host tissues, facilitating the spread of infection throughout the host (13). Cells in wild-type C. *albicans* biofilms are normally either hyphal form or yeast form cells, but occasionally pseudohyphal cells have been observed (33, 70).

C. *albicans* biofilm formation has been viewed as a stepwise process (Fig. 2) based mainly upon in vitro experiments (22, 33). While these steps may occur concurrently in natural biofilms, they provide a useful way to organize and understand biofilm formation. Biofilm formation begins with adherence to a substrate and the proliferation of yeast form cells across the surface (adherence step) (Fig. 2A). Soon afterward, the yeast form cells begin to grow as hyphae (Fig. 1C). Production of extracellular matrix material begins, and this material accumulates as the biofilm matures (biofilm initiation step) (Fig. 2B). The biofilm mass increases as hyphal cells continue to grow and increased extracellular matrix is produced. At this point yeast cells make up the basal layer of cells attached to the substrate and are surrounded by hyphal cells and extracellular matrix (mature biofilm step) (Fig. 2C). Finally, many recent studies suggest that there may be a final step in the C. *albicans* biofilm development, a dispersal stage in which daughter cells bud as nonadherent yeast cells and are released from the biofilm into the surrounding medium (dispersion step) (Fig. 2D). Although the steps may occur concurrently or vary during actual infection in vivo, the pathway is a beneficial way to organize biofilm formation and has been useful for identification of key functions that operate in vivo (103, 105, 106).

THE GENETIC CONTROL OF BIOFILM DEVELOPMENT

Many different kinds of approaches—expression profiling, heterologous expression, mutant screening, and some good guesses—have identified genes that participate in biofilm formation in vitro (Table 1). The discovery of these genes allowed definition of the genetic pathways for biofilm formation. We restrict our attention here to (i) genes in which mutations reduce or abolish biofilm formation, (ii) genes in which mutations alter biofilm mass or structure, (iii) genes whose altered expression can rescue biofilm defects, or (iv) genes that are connected specifically to the increased drug resistance of biofilms. We include mutations that cause loss of function as well as those that increase expression. Many additional genes are implicated in biofilm formation through

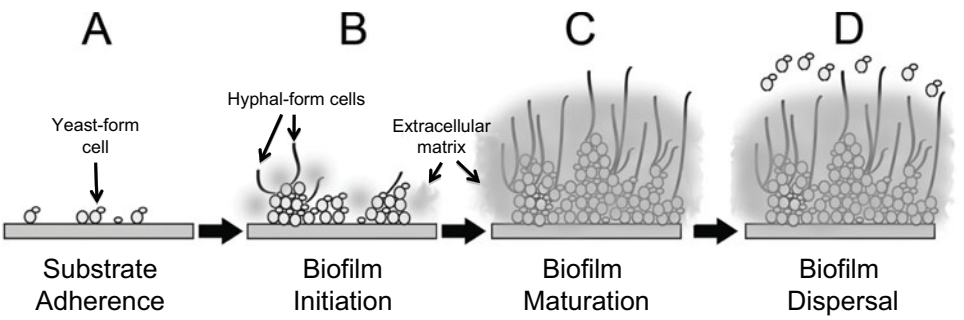

FIGURE 2 Schematic diagram of the different steps of in vitro biofilm development. (A) In the first step, yeast form cells adhere to the substrate. (B) In the second step, the cells multiply and accumulate. Germ tubes begin to form, and extracellular matrix production begins. (C) In the third step, the biofilm enlarges, with increased cell density, yeast cells, and hyphal cells. Extracellular matrix production increases, enveloping the biofilm. (D) In the final step, yeast cells are dispersed to further colonize the surrounding environment. 10.1128/9781555817176.ch19f2

TABLE 1 Genes that govern *C. albicans* biofilm formation

Gene	Molecular function of gene product[a]	Mutant phenotype[b]	Net role of gene product[c]	Reference(s)
ACE2	TF	*ace2-*: severe defect in biofilm formation; aberrant hyphae	Positive	60
ADH1	Alcohol dehydrogenase	*adh1-*: increased biofilm biomass	Negative	97
ADH5	Alcohol dehydrogenase	*ADH5-oe*: increased biofilm matrix	Positive	106
ALS1	CWP	*als1- als3-*: severe defect in biofilm formation *ALS1-oe*: restores biofilm in *bcr1-*	Positive	103, 107
ALS2	CWP	*als2-down*: defect in biofilm formation and hypha formation. *ALS2-oe*: partially restores biofilm formation in *als1- als3-*	Positive	107, 152
ALS3	CWP	*als3-*: reduced or altered biofilm formation *als1- als3-*: severe defect in biofilm formation in vitro *ALS3-oe*: restores biofilm in *bcr1-* and in *efg1-*	Positive	103, 107, 151
ALS4	CWP	*ALS4-oe*: partially restores biofilm formation in *als1- als3-*	Positive	107
ALS5	CWP	*ALS5-oe*: partially restores biofilm formation in *als1- als3-*	Positive	107
ALS7	CWP	*ALS7-oe*: partially restores biofilm formation in *als1- als3-*	Positive	107
ALS9	CWP	*ALS9-oe*: partially restores biofilm formation in *als1- als3-*	Positive	107
BCR1	TF	*bcr1-*: severe defect in biofilm formation *BCR1-oe*: partially restores biofilm in *tec1-*	Positive	103, 104
CBK1	PK	*cbk1-*: severe defect in biofilm formation and hypha formation	Positive	17
CHK1	Histidine kinase	*chk1-*: forms farnesol-resistant biofilm	Negative	67
CSA1	CWP	*csa1-*: defect in biofilm formation; additive with *pga10-* and *rbt5-*	Positive	117
CSH1	Aryl-alcohol dehydrogenase	*CSH1-oe*: decreased biofilm matrix in *zap1-*	Negative	106
CZF1	TF	*czf1-*: defect in biofilm formation; aberrant hyphae	Positive	71
EAP1	CWP	*eap1-*: severe defect in biofilm formation	Positive	84
ECE1	Putative transmembrane protein	*ECE1-oe*: restores biofilm in *bcr1-*	Positive	103
EFG1	TF	*efg1-*: severe defect in biofilm formation and hypha formation	Positive	82, 122
FLO8	TF	*czf1-*: defect in biofilm formation; aberrant hyphae	Positive	20
GCA1	Glucoamylase	*GCA1-oe*: increased biofilm matrix	Positive	106
GCA2	Glucoamylase	*GCA2-oe*: increased biofilm matrix	Positive	106
GCN4	TF	*gcn4-*: reduced biofilm biomass	Positive	39
GIN4	PK	*gin4-*: severe defect in biofilm formation; aberrant hyphae	Positive	17
HWP1	CWP	*hwp1-*: defect in biofilm formation and hyphal formation HWP1-oe: restores biofilm in *bcr1-*	Positive	103, 105
IFD6	Aryl-alcohol dehydrogenase	*IFD6-oe*: decreased biofilm matrix in *zap1-*	Negative	106
IRE1	PK	*ire1-*: severe defect in biofilm formation and hypha formation	Positive	17
KEM1	Exo-RNAse	*kem1-*: severe medium-dependent defect in biofilm formation and hypha formation	Positive	124

(continued on next page)

TABLE 1 Genes that govern *C. albicans* biofilm formation *(Continued)*

Gene	Molecular function of gene product[a]	Mutant phenotype[b]	Net role of gene product[c]	Reference(s)
MDS3	Unknown	*mds3-*: severe medium-dependent defect in biofilm formation and hypha formation	Positive	124
MKC1	MAP kinase	*mkc1-*: aberrant biofilm, with reduced filamentation	Positive	69
NUP85	Nuclear pore protein	*nup85-*: severe medium-dependent defect in biofilm formation and hypha formation	Positive	124
OCH1	Alpha-1,6-mannosyltransferase	*och1-*: biofilm defect; cellular aggregation, cell wall defects	Positive	11
PEP12	Vacuolar trafficking	*pep12-*: biofilm defect, fragile biofilm, reduced biomass, and abnormal hyphae	Positive	112
PGA10	CWP	*pga10-*: defect in biofilm formation; additive with *rbt5-* and *csa1-*	Positive	117
PMT1	Mannosyltransferase	*pmt1-*: biofilm defect reduced mass; aberrant hyphae	Positive	116
PMT2	Mannosyltransferase	*pmt2-/+*: biofilm defect (reduced biomass); aberrant hyphae	Positive	116
RBT5	CWP	*rbt5-*: defect in biofilm formation; additive with *pga10-* and *csa1-* *RBT5-oe*: restores biofilm in *bcr1-*	Positive	103, 117
SUN41	CWP	*sun41-*: severe defect in biofilm formation; aberrant hyphae	Positive	108
SUV3	Mitochondrial RNA helicase	*suv3-*: severe medium-dependent defect in biofilm formation and hypha formation	Positive	124
TEC1	TF	*tec1-*: severe defect in biofilm formation and hypha formation	Positive	104
UME6	TF	*ume6-*: reduced-mass biofilm; aberrant hypha formation	Positive	10
YAK1	PK	*yak1-*: severe defect in biofilm formation and hypha formation	Positive	41
YWP1	CWP	*ywp1-*: increased biofilm formation by yeast form cells	Negative	42
ZAP1	TF	*zap1-*: increased biofilm matrix	Negative	106

[a]Molecular functions have been inferred from protein sequence homology in most cases. Abbreviations: CWP, cell wall protein; PK, protein kinase; TF, transcription factor.

[b]Mutant phenotype was observed with either homozygous loss-of-function alleles (e.g., *als1-* refers to an *als1/als1* homozygous mutant) or overexpression alleles (e.g., *ALS1-oe* refers to a strain in which one *ALS1* allele has been fused to a strong promoter). The "*als2-down*" strain had reduced but not abolished *ALS2* expression. Negative results (absence of a phenotype) are omitted.

[c]"Net role of gene product" is a tentative assignment based on current genetic information. A "positive" role reflects either a biofilm defect caused by a loss-of-function mutation or augmented biofilm formation caused by an over-expression allele. A "negative" role reflects either augmented biofilm formation caused by a loss-of-function mutation or a biofilm defect caused by an overexpression allele.

gene expression and protein accumulation studies, but discussion of their possible functional roles would be quite speculative at present.

Inspection of Table 1 reveals that the 44 genes listed fit into a few general categories that help to visualize genetic relationships. First, we have indicated whether genes have net positive or negative roles in biofilm formation, based on known mutant phenotypes. A positive role indicates that the gene acts to favor biofilm formation, and a negative role indicates that the gene functions to inhibit biofilm formation. These tentative assignments are for simplicity's sake based upon the first three steps in biofilm formation shown in Fig. 2, because the "dispersal" stage can be viewed as a local dismantling of the biofilm. Hence, a gene with a negative role in "initiation" may also have a positive role in "dispersal." These general functional categories are useful in thinking about gene relationships because in theory, a negative gene product may act by inhibiting expression or activity of a positive gene product. Second, many of the genes are required for production of hyphae, or filamentation. Hyphae are prominent features in *C. albicans* biofilms (Fig. 1C), and mutant studies long ago indicated that hyphal formation is required for stable biofilm formation (9, 122). Third, we identify genes involved in response to the quorum-sensing molecule farnesol (30). Farnesol is an inhibitor of hyphal formation and, as might be expected, an inhibitor of biofilm formation. It has been shown that farnesol and other quorum-sensing molecules accumulate in biofilms, where they probably promote dispersal of mature biofilms (2, 40). Fourth, many of the genes encode known or predicted cell wall proteins, and it seems reasonable that they play a direct role in cell-substrate or cell-cell adherence. Heterologous

expression studies have shown Epa1 and Hwp1 indeed have such roles (83, 107). In addition, several Als family members bind to diverse substrates (21, 34) and to each other (64, 111). Fifth, many of the genes encode known transcription factors and protein kinases. These genes are important since biofilm formation is a pathway: the expression of certain genes and enzymatic activity of some proteins will need to be tightly regulated. Sixth, several alcohol dehydrogenases and aryl-alcohol dehydrogenases have either positive or negative roles in biofilm formation (97, 106).

Based upon mutant phenotypes and regulatory relationships, we can begin to assemble a model that relates gene function to specific features of biofilm formation (Fig. 3). We present the following model as a framework for planning experiments and interpreting proposed gene function in biofilm formation.

EARLY FUNCTIONS: ADHESION AND SURFACE RESPONSE

In the initial step of biofilm formation, yeast form cells bind to a substrate and begin to aggregate. Questions abound as to whether *C. albicans* detects that a substrate is available for adhesion or whether the cells are always ready to adhere with anything in their environment. The *C. albicans* cell is surrounded by a rigid wall that lacks appendages that would be used to sense available substrates. Regardless of this lack of sensory organelles, by 30 min after contact with a polystyrene surface a cascade of gene expression is initiated that is distinct from that seen in planktonic cells grown under similar conditions (99).

The adherence of yeast form cells to the substrate is the first step in biofilm formation. To date only one gene, *YWP1*, has been identified that specifically regulates this crucial initial step (42). Ywp1 is a wall mannoprotein that is specifically transcribed in yeast form cells. Microarray analysis showed that *YWP1* expression is dependent on Efg1, but only in yeast form cells, as little to no *YWP1* transcript is found in hyphal cells regardless of the genotype (131). Ywp1 appears to inhibit both adhesion and biofilm formation by yeast form cells (also called blastoconidia). The work of Granger et al. indicates that Ywp1 may act as an antiadhesin (42). Deletion of *YWP1* leads to greatly enhanced adherence of yeast cells to a number of surfaces (42). Both a Ywp1 propeptide and extracts from cultures of Ywp1-expressing stationary-phase cells inhibit adherence of strains not expressing Ywp1, suggesting that the Ywp1 propeptide can inhibit adherence in surrounding cells (42). To date *YWP1* is the only known gene to affect biofilm formation that is expressed only in yeast form cells. While specific to yeast form cells, Ywp1 has been posited to function in biofilm dispersal. It is hypothesized that as the biofilm matures it releases yeast form cells to further colonize the host. This release requires the reduction of adhesion in the yeast form cells so that they can be dislodged or released from the biofilm.

It is reasonable to assume that if there is an inhibitory response to biofilm formation, there must also be an activator

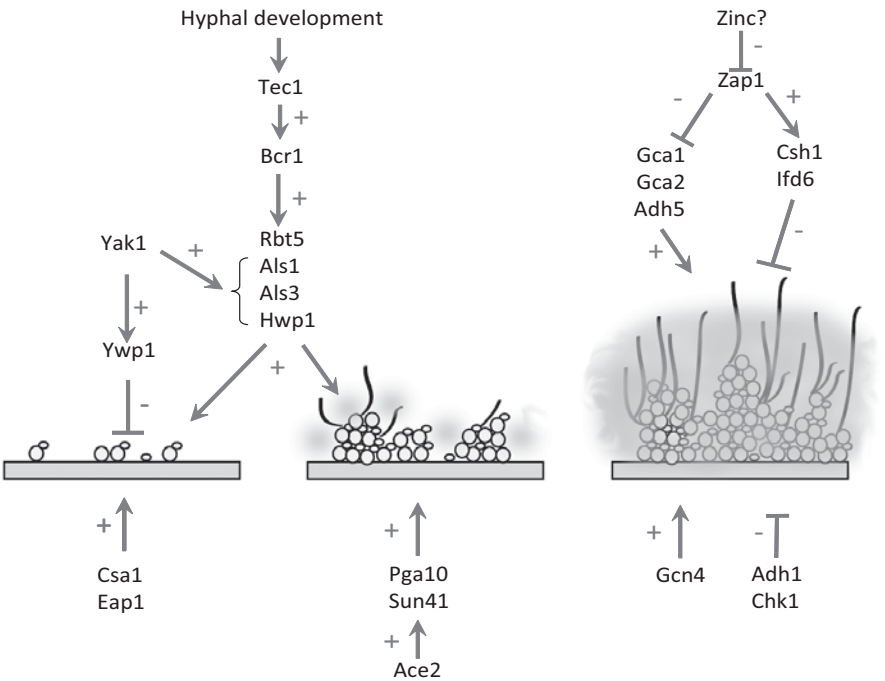

FIGURE 3 Graphic representation of known pathways involved in biofilm formation. The first three steps of biofilm formation are presented. Arrows with a plus sign represent positive genetic control. "T" bars with a minus sign represent inhibitory action by the protein(s) on the particular step. Those proteins not associated with a particular pathway are presented as follows in the category that best represents their hypothesized function: adhesion, Gin4, Ire1, and Och1; biomass, Als2, Pep12, Pmt1, and Ume6; filamentation, Cbk1, Efg1, Flo8, Kem1, Mds3, Mkc1, and Suv3; and rescue of biofilm defect, Als3, Als5, Als7, Als9, Czf1, and Ece1. 10.1128/9781555817176.ch19f3

response that encourages the initial adhesion and then propagation of the biofilms. This possible activation may be the response to environmental conditions that are best suited for biofilm formation. Most likely adhesins or cell wall proteins act to trigger the yeast cells to bind in favorable conditions. Shortly after adhesion to the substrate, the expression of efflux pump genes *CDR1* and *MDR1* is activated (69). The rapid response to adherence indicates that the cell undergoes a rapid, substrate-responsive change from planktonic free-floating yeast to yeast that may become biofilms. Additional genes are induced early in biofilm formation, including several methionine and cysteine biosynthetic genes. In addition, a number of genes shown to be essential for biofilm formation are either repressed or upregulated in response to pH, temperature, and hypoxic and nutrient conditions (10, 27, 28, 106, 137).

MIDDLE FUNCTIONS: BIOFILM INITIATION, FILAMENTATION, AND CELL-CELL ADHESION

Adherence of *C. albicans* cells both to solid substrates and to other cells is vital for biofilm formation. Biofilm growth is initiated when yeast form cells (Fig. 1A) adhere to a surface, followed by cell-cell attachments, which allow the biofilms to become layered, differentiated biomasses (Fig. 2). It is not surprising that a number of identified adhesin and cell wall genes are required for early biofilm formation. Cell wall genes and adhesins provide mechanisms that promote biofilm initiation, and the transcription factors that regulate their expression couple biofilm initiation with internal and external signals. The study of transcription factors has laid the framework for gene regulatory networks. Two transcription factors regulate a part of early biofilm formation, Bcr1 and Tec1 (104).

Tec1 and Bcr1 were identified in a screen of transcription factor mutants for biofilm defects (104). Tec1 is a TEA/ATTS transcription factor that is required for hypha formation and virulence (126). A potential binding site for Tec1 has been identified in upstream sequences of all known hypha-specific genes, indicating a strong role for Tec1 in hypha formation. The transcription of Tec1 is regulated by Cph2, which binds to a conserved sequence upstream of Tec1 (78). Tec1 regulates the transcription of *BCR1*, which, in turn, regulates genes downstream of hypha formation. A *tec1/tec1* insertion mutant is unable to undergo hyphal morphogenesis and can form only a rudimentary biofilm. This rudimentary biofilm is composed entirely of yeast form cells and is at least 10-fold smaller than the biofilm formed in a *TEC1/TEC1* strain (104). A biofilm composed of only yeast form cells indicates that while the first step of the biofilm formation pathway can occur, the inability to produce hyphal cells bars the biofilm from maturing into a full biofilm. This highlights the requirement of hyphal form cells in the creation of a mature biofilm.

One major gene regulatory network that governs early biofilm formation has been dissected through the study of Bcr1, a zinc finger transcription factor (104). Interestingly, the *C. parapsilosis* Bcr1 ortholog is also a regulator of biofilm formation (32), suggesting extensive functional conservation. In *C. albicans*, a *bcr1/bcr1* mutant fails to produce a mature biofilm and produces only a rudimentary biofilm composed of yeast and hyphal cells. In contrast to most biofilm-defective mutants, *bcr1/bcr1* strains are able to form hyphae efficiently under numerous conditions. The mechanistic role of Bcr1 was initially indicated through microarray analysis (104). Bcr1 is required for expression of numerous genes, including *ALS1*, *ALS3*, *HWP1*, and *RBT5*, all of which specify cell surface proteins that are known to govern adhesion in some contexts (104). Mutant analysis showed that Als3 is important for biofilm formation in vitro but is dispensable for biofilm formation in vivo (103, 151), whereas Hwp1 has a role in biofilm formation in vitro and in vivo (105). Overexpression of either gene can at least partially rescue the biofilm defect of a *bcr1*Δ*/bcr1*Δ mutant strain in vitro and in vivo (103, 105), providing further evidence that these genes work downstream of Bcr1 in promoting biofilm formation. Other Bcr1 downstream targets may contribute to overall biofilm development, but mutants with mutations in these target genes show minimal biofilm defects in vitro (103). Strains lacking Tec1, Bcr1, Als3, or Hwp1 demonstrate severe biofilm defects, which suggest that they are important for early events in biofilm formation, but the fact that these mutants all produce a thin rudimentary biofilm suggests that these proteins may not be absolutely required in yeast form cell surface adherence (103–105, 151). Their likely role is in early cell-cell adherence events, and because *ALS3* and *HWP1* are expressed only in hyphae, they probably mediate hypha-hypha or hypha-yeast adherence (55, 136). In addition, the fact that the accumulation of *BCR1* RNA is greatest in hyphal cells suggests that the hyphal regulators Cbk1, Efg1, Kem1, Mds3, Nup85, Suv3, and Tec1 may be required for biofilm formation primarily to activate *BCR1* expression as hyphae form. This event occurs after the initial step of yeast form cell adhesion to the substrate.

HWP1 encodes a developmentally regulated adhesin, found on surfaces of germ tubes but not yeast form cells or pseudohyphae of *C. albicans*. Hwp1 is a substrate for mammalian transglutaminases and becomes cross-linked to transglutaminase-expressing surface squames of the oral mucosa (135, 136, 139). While *HWP1* is not expressed in yeast form cells, it is strongly expressed in hyphal form cells (100, 129, 135, 136). *hwp1/hwp1* insertion mutants create biofilms with a threefold decrease in biomass compared to that of a wild-type strain. The biofilm produced is approximately half the size of wild-type biofilms and contains very few hyphal cells. In the medium surrounding the biofilm there is a dramatic increase of cells compared to the wild type, suggesting that Hwp1 is required for cell retention within the biofilm. These observations are consistent with the hypothesis that Hwp1 is an adhesin that increases cell-cell interactions within the biofilm (105). Since a rudimentary biofilm is comprised primarily of yeast cells, Hwp1 function in biofilm formation occurs after the initial adherence step.

The eight agglutinin-like sequence (Als) proteins have long been considered excellent candidates for biofilm adhesins. The Als proteins are large cell surface glycoproteins. Expression of several *ALS* genes is upregulated during biofilm formation (44). In vivo studies have shown that deletion of either *ALS1 or ALS3* does not abolish biofilm formation, but a double deletion strain has a strong biofilm defect. This indicated an overlap of the roles of *ALS1 and ALS3* in biofilm formation. In fact, overexpression of either *ALS1 or ALS3* was able to partially rescue the biofilm defect seen in a *bcr1*Δ*/bcr1*Δ strain (103, 107). The shared function of Als1 and Als3 is consistent with their similarity in sequence (88% amino acid identity of their N-terminal 772 residues) and their predicted N-terminal domain structures (130). Further examination for the possible overlap in function in the other *ALS* genes revealed that in an *als1*Δ*/als1*Δ *als3*Δ*/als3*Δ strain, overexpression of *ALS6*, *ALS7*, or *ALS9* can

rescue the biofilm defect. Overexpression of *ALS5* in the same strain was only able to moderately rescue the biofilm defect. These studies indicate that there may be a large overlap in the functions of the Als proteins (107). It may be that the expression levels determine which Als protein is functional at any given time. This hypothesis is based on the facts that *ALS3* is upregulated during hyphal morphogenesis and that *ALS1* has increased expression during biofilm formation (22, 55, 109). Overexpression of *ALS2* and *ALS4* was unable to rescue the biofilm defect in the *als1*Δ/*als1*Δ *als3*Δ/*als3*Δ strain, indicating that they may function in a role separate from those of the other Als proteins (103). However, recent studies indicate that the Als2 and Als4 proteins are required in biofilms and may thus have a role distinct from those of other Als proteins. Zhao et al. found that a reduction in Als2 expression causes a reduction in biofilm biomass (152). In addition, strains with reduced Als2 expression or lacking Als4 showed decreased adhesion to vascular endothelial cells. The lack of a complete biofilm defect suggests that Als2 and Als4 contribute to later stages of biofilm development and not to initial adherence steps. However, this inference is limited by the fact that Als2 expression was reduced but not abolished, since the protein appears to be essential (152). Thus, Als2 may have a greater role in biofilm formation than can be deduced by mutant analysis, given its unexpected role in viability. Als2 and Als4 contain a conserved tandem repeat domain and a 3′ domain that is more than 95% identical between the genes (54). This similarity may mean that to uncover the specific roles of Als2 and Als4, the creation of double mutants is required. Such double-mutant analysis has proven very insightful for other highly homologous genes, such as those for Als1 and Als3. Since Als1 and Als3 have been identified to function in hyphal adhesion, and overexpression of Als2 or Als4 is unable to rescue the mutant phenotype, the precise role of Als2 and Als4 may be to regulate a different form of adhesion. They may be responsible for specific cell-cell adhesion that occurs within the biofilm and/or involved in the dispersal stage, a stage for which there is not yet a clear assay to identify acting proteins. The reduction of biomass in the mutant *als2/als2* strain would indicate a function in the overall structure of the biofilm.

Eap1 is a member of the family of glycosylphosphatidylinositol-anchored cell wall adhesins required for cell-cell adhesion. Deletion of *EAP1* results in reduced adhesion to polystyrene and epithelial cells, and reduced cell-cell adhesion when grown under shear flow (84). While adhesion is reduced, it is not abolished, indicating an *EAP1*-independent mechanism also exists for the cell surface adhesion. Li et al. used a unique system where *C. albicans EAP1* was expressed in an adhesion-deficient *S. cerevisiae* strain and tested for the ability of cell-cell adherence in a shear flow system (85). Their results showed that when *C. albicans EAP1* is expressed, the amount of cells that are able to maintain their cell-cell adherence doubles compared to the vector control under low-flow conditions, whereas under high-flow conditions, these cell-cell adhesion events were maintained over 25-fold greater in those cells expressing *C. albicans EAP1* than in the vector (85). Both in vitro and in vivo *eap1/eap1* mutants are unable to form a biofilm, with almost no cells adhering to the substrate, reflecting that the defect is in cell-cell and cell-substrate adhesion. *EAP1* is unusual in that its expression levels are similar in the yeast form and hyphal form cells, indicating that Eap1 may function in similar ways in the two cell types (85). In biofilms *EAP1* expression is approximately double that observed under planktonic conditions, indicating a strong role for Eap1 in biofilm formation and maintenance. Since *eap1/eap1* mutants do not disrupt filamentation, these results indicate that the affect of *EAP1* on adhesion and biofilm formation is independent of filamentation and that adhesion and filamentation contribute to biofilm development separately (83, 85). *EAP1* represents a gene that has multiple functions within a biofilm. It is involved in the initial adherence of the cells to the substrate, and as the biofilm matures, its expression increases, indicating that it functions within the biofilm to maintain the structural cohesiveness of the biofilm.

The cell wall of *C. albicans* contributes to pathogenicity through adherence and invasion and is a target of both chemical and immunological antifungal strategies. *SUN41* plays major roles in cell wall integrity, biofilm formation, and virulence in both oropharyngeal and disseminated candidiasis (51, 108). A *sun41/sun41* mutant can produce elongated cells, but the cell walls are not uniformly parallel and have the appearance of being an intermediate between hyphae and pseudohyphae (51, 108). A *sun41/sun41* mutant has a severe biofilm defect in vitro, with the biofilm appearing to be unable to stay adhered to the substrate and detaching into the reservoir medium (108). The defect is distinct from that seen with a *bcr1/bcr1* deletion mutant or a *tec1/tec1* insertion mutant, where the bulk of cells grow in suspension in fairly small cell aggregations (51, 108). The exact role of *SUN41* in biofilm formation is unknown, but gene expression data suggest that Sun41 function has an impact on cell wall integrity (51, 108). Thus, a *sun41/sun41* mutant may have a global defect in cell wall integrity that then compromises adhesin function, resulting in the observed biofilm defect (108).

Biofilm formation is a complex biological process that requires fungal cells to establish multiple interactions with the environment. One promising family of proteins that may function in this interaction is the fungal proteins containing an eight-cysteine domain referred to as CFEM (common in several fungal extracellular membrane proteins). CFEM proteins are posited to function as cell surface receptors, signal transducers, or adhesion molecules in host-pathogen interactions (68). In *C. albicans* there are five members of the CFEM protein family: Csa1, Csa2, Rbt5 (a Bcr1 target), Pga7, and Pga10 (an Ace2 target) (145). *pga10/pga10* mutants are able to form an apparently normal biofilm during the first few hours, but the biofilm detaches from the plastic substrate after 12 h of incubation, in contrast to biofilms formed by the wild-type strain (117). This detachment indicates a role for Pga10 in biofilm maintenance and adherence. Deletions of *RBT5* and *CSA1* also resulted in biofilm defects similar to that seen in a *pga10/pga10* strain (117). *CSA1* is upregulated during hyphal production but is localized predominately to growing bud surfaces in exponentially growing yeast cultures. In hyphae, Csa1 is localized along the germ tube but not to the parent blastospore. Taken together, these findings indicate that Csa1 is restricted to the site of cell surface elongation and may have a role in cell-cell adhesion (76).

The ability to switch from yeast to hyphae is one of the most important requirements for virulence. As a cell transitions from yeast to hyphae, multiple changes in the cell wall are required, including an increase in chitin levels in hyphae (98). Chitin provides skeletal support to the yeast and hyphal cell wall and is regulated using both chitin synthase and chitinase enzymes (60). The regulation of chitin levels is best understood in *Saccharomyces cerevisiae*, where Ace2

regulates a single chitinase gene, *CTS1*. *ACE2* is expressed during the cell cycle G_2 phase and is found only in the daughter cell. In *C. albicans*, *ACE2* is a regulator of one of the chitinase genes, *CHT3* (60). Cells of an *ace2*/*ace2* mutant fail to separate following cell division and have an increased tendency to form pseudohyphae (60). An *ace2*Δ/*ace2*Δ strain has a moderate adherence defect and is incapable of production of a confluent biofilm in vitro. *C. albicans* Ace2, like its *S. cerevisiae* homolog, governs expression of several cell wall protein genes. It is required for full expression of numerous cell surface protein genes, notably *PGA10* and *SUN41* (21). Both Pga10 and Sun41 are required for biofilm formation and are known to govern adhesion. The loss of Ace2 mimics the defects seen in *sun4*/*sun41* and *pga10*/*pga10* mutants, indicating a role of regulation for Ace2 in both genes. This role may be to control the cell-cell adhesion within the biofilm.

Another factor identified in the transition from yeast cells to hyphae is *EFG1*. *EFG1* is a transcription factor with a basic helix-loop-helix motif (138). Efg1 is an essential regulator of morphogenesis in *C. albicans* and is a member of a conserved class of basic helix-loop-helix (bHLH) proteins (133, 134). *EFG1* expression is induced during filamentation and is essential for the production of hyphal cells. *efg1*/*efg1* mutants fail to form biofilms, producing instead a sparse monolayer of short, elongated cells that are unable to form hyphae (122). The fact that yeast cells still attach indicates that Efg1 is not involved in the initial step of adherence but has a role either in the cell-cell adherence or in the transition from yeast form cells to hyphal form cells. Further evidence for a direct role of Efg1 in biofilm formation came by DNA microarray analysis, which identified a major role for Efg1 in the repression and induction of a number of cell wall genes. Efg1 regulates cell wall genes that are both yeast form specific (*YWP1*) and hyphal form specific (*HWP1*, *HYR1*, *HWP2*, and *PGA10*) (77, 131). In total, the data indicate a major role for Efg1 in the initiation and maintenance of the morphological switch from yeast cells to hyphae. Since the morphological switch is essential for wild-type biofilm formation, Efg1 is a major regulator of biofilm formation.

The transcription factor *CZF1* controls contact-dependent filamentation. A *czf1*/*czf1* mutant can filament under most conditions but is defective in its ability to induce hyphae upon embedding in a matrix (19). *czf1*/*czf1* mutants form biofilms with reduced mass and reduced metabolic activity (127, 137). Interestingly, Czf1 is required for the formation of true hyphae in hypoxic surface growth at temperatures less than 37°C (142). There is an intricate series of pathways that govern the contact response of both yeasts and hyphae. These contact pathways are essential for biofilm formation. Both contact events are dependent on environmental conditions such as temperature and nutrients (137). The pathways allow the biofilm to adapt to the environmental conditions of its localization. This ability to adapt is essential for an organism, such as *C. albicans*, that colonizes such a diverse set of environments, and it is a reason for the persistence of and difficulty in eradicating biofilms.

The ability of *C. albicans* to interact with its external environment is essential to its survival. The cell surface functions in this interactive role by both sensing external signals and binding to other cells or external surfaces (17). The ability of the cell to bind to a substrate and bind to other cells is essential for the formation of biofilms. When a cell comes into contact with another cell or substrate, this interaction creates a signal pathway that triggers the cell to react to this interaction (69). Signaling pathways are often mediated by protein kinases. In a screen of protein kinase-defective mutants, Blankenship et al. found that disruptions in *CBK1*, *IRE1*, and *GIN4* result in strains with increased sensitivity to cell wall stress and a failure to form hyphal cells under filamentation-inducing conditions. All three mutant strains cause severe defects in biofilm formation. The biofilms formed in these mutant strains have displaced aggregation of cells in the surrounding medium (17). These protein kinases in biofilm formation can be hypothesized to initiate hypha formation within the biofilm. Since filamentation is required for biofilm formation, the failure to initiate hyphal growth results in an inability to form a biofilm (9, 122). In addition, these genes may have a role in activating adhesion elements within the cell. This adhesion triggered may be essential for hypha-yeast cell, hypha-hypha, and/or hypha-substrate adhesion.

The protein kinase Yak1 is required early in biofilm formation. Yak1 is a member of the family of dual-specificity tyrosine phosphorylation-regulated kinases (DYRK). The DYRK family differs from other kinases by its activation mechanism. The activity of the DYRK kinase depends on tyrosine autophosphorylation at a conserved YxY site in the catalytic domain. While DYRK kinases are able to autophosphorylate at a tyrosine residue, mature phosphorylated members have only serine/threonine kinase activity (86). In *C. albicans*, a *yak1*/*yak1* mutant produces a biofilm with few hyphal cells and a fivefold decrease in biomass compared to the wild-type strain (41). While defective in biofilm formation, a rudimentary biofilm forms and no significant difference is observed in initial adherence compared to a wild-type strain. Interestingly, while not required for expression of *BCR1* or *TEC1*, it is required for expression of some Bcr1-dependent adhesin genes, including *ALS1*, *ALS3*, and *HWP1*, though not *RBT5* (41). These genes are all expressed preferentially in hyphae and are thought to be positive regulators of biofilm formation. Yak1 also inhibits the expression of the cell wall protein gene *YWP1*, which is expressed preferentially in yeast form cells as described above (41). These genes are all expressed preferentially in hyphae and are thought to be positive regulators of biofilm formation. Yak1 also inhibits the expression of the cell wall protein gene *YWP1*, which is expressed preferentially in yeast form cells as described above (41). The fact that Yak1 is required both for the expression of adhesin genes *ALS1*, *ALS3*, and *HWP1* and for repression of the antiadhesin gene *YWP1* provides increased evidence for Yap1 being a positive regulator of biofilm formation.

Our understanding of the roles of adherence factors and factors that induce hypha formation in biofilm formation is building with the identification of genes involved in cell-cell adherence. While much is now known, detailed testing of other known adherence factors, cell wall proteins, transcription factors, kinases, and others is required to identify more genes involved in biofilm formation, and the elucidation of upstream regulation of these factors will allow for greater insights into the signaling events important for biofilm development.

LATE FUNCTIONS: INCREASED DENSITY, EXTRACELLULAR MATRIX, AND CELL DISPERSAL

As the biofilm matures, increased hyphal growth is paired with increased extracellular matrix production. As maturation continues, the biofilm grows in both size and mass.

Here several known gene products are assigned conceptually as maturation functions that are not connected to adherence yet clearly affect overall biofilm biomass, metabolic activity, stability, or extracellular matrix production.

The production of a biofilm requires numerous components, including nutrients and amino acids. The transcription factor Gcn4 functions as an activator of amino acid biosynthetic genes. A *gcn4*/*gcn4* mutant results in a biofilm of reduced biomass (140). This phenotype suggests that Gcn4 may be required for overall metabolic activity in the biofilm. Not surprisingly, Gcn4 also stimulates hyphal development in response to amino acid limitation (140). The lack of amino acids in a *gcn4*/*gcn4* mutant may result in a biofilm that is unable to make all of the required proteins essential for biofilm formation. The lack of amino acids may result in an immature biofilm that, while able to form, is unable to form a true mature biofilm, because of the lack of materials to complete the biofilm.

One of the defining characteristics of all biofilms is the presence of an extracellular matrix (Fig. 2). The extracellular matrix is composed of carbohydrates, proteins, phosphorus, glucose, and hexosamines, but a large portion of the matrix is not molecularly well defined (8). The extracellular matrix is thought to contribute to the architectural preservation of biofilms by the maintenance of stable cell-cell and cell-surface interactions and acts as a protective barrier (3, 4, 102). In addition, this complex extracellular material may function to defend against phagocytic cells, to serve as a scaffold for maintaining biofilm integrity, to limit diffusion of toxic substances into the biofilm, or some combination of the above.

In an attempt to identify genes involved in extracellular matrix production Mukherjee et al. used two-dimensional gel electrophoresis with samples from planktonic and early biofilm cultures, differentially labeled, to find soluble cell wall/matrix proteins expressed at different levels in these cultures (97). They identified 24 differentially expressed proteins that are downregulated in early biofilms, including Adh1 (97). Adh1 is an alcohol dehydrogenase that catalyzes the production of ethanol from acetaldehyde. Adh1 levels were higher in the planktonic sample than in the biofilm sample, suggesting that Adh1 may be an inhibitor of biofilm formation (97). This hypothesis of biofilm inhibition is strengthened by properties of an *adh1*/*adh1* insertion mutant, which produces a biofilm of increased thickness and increased metabolic activity relative to its parent strain or to an *adh1*/*adh1*/*ADH1* complemented strain. Biofilms grown in inhibition studies were treated with disulfiram and 4-methylpyrazole, each of which targets alcohol dehydrogenase enzyme activity. Treated biofilms had increased thickness, indicating that the critical Adh1 function is its enzymatic activity (97). Those biofilms grown in the presence of disulfiram had an elaborate network of *C. albicans* hyphae enmeshed in a carbohydrate-rich extracellular matrix. These studies provide pharmacological confirmation that Adh1 restricts the ability of *C. albicans* to form biofilms. Indeed, inhibition of Adh1 activity chemically or by gene disruption resulted in more robust biofilm production in vitro (97). Additionally, Adh1 was identified as one of eight *C. albicans* cell wall-associated proteins able to bind plasminogen and to activate plasmin release. Since plasmin has proteolytic activity, and an *adh1*/*adh1* mutant has a greater ability to penetrate human oral mucosa layers, it is possible that Adh1 may modulate host tissue invasion by cells released from a biofilm (25, 97). Furthermore, Adh1 binds to antibodies that recognize the cell wall, indicating that Adh1 may mediate cell-cell or cell-surface interactions (65). Thus, the function of Adh1 is complex and involves multiple roles for Adh1 in biofilm formation: its enzymatic activity is probably manifested intracellularly (due to absence of extracellular NAD^+ or NADH), and it may also affect cell surface properties directly (65).

The transcription factor Zap1/Csr1 is a net negative regulator of biofilm matrix production (Fig. 2). Prior to studies done by Nobile et al. (106), the transcription factor Zap1 was indicated in the regulation of zinc metabolism (62, 63). Zinc is an essential nutrient that functions as a structural component of the zinc finger motif found in many transcription factors and as a catalytic cofactor for many enzymes in yeast and humans (14, 15). Thus, zinc ions are essential for growth and viability, and their import is tightly regulated. In a *zap1*Δ/*zap1*Δ strain, biofilm formation occurs normally except that the biofilms appear "slimier" (106). Yeast cells in these biofilms are swollen, and there is an increased amount of extracellular matrix within the biofilm. In the *zap1*Δ/*zap1*Δ strain, levels of soluble β-1,3 glucan, the most abundant component of the matrix, are significantly elevated compared to those in the complemented and reference strains (106). This increase in soluble β-1,3 glucan is not observed under planktonic conditions, indicating that this phenotype is specific to biofilms. Microarrays performed on biofilms from a *zap1*Δ/*zap1*Δ *C. albicans* strain and the complemented strain identified a large number of genes that are regulated by Zap1 (106). A set of unique target genes were identified that were unrelated to the zinc transporter or to homeostasis and fixation genes previously identified as being targeted by Zap1. The genes were overexpressed in both *zap1*Δ/*zap1*Δ and *ZAP1*/*ZAP1* backgrounds to detect both positive and negative functions in matrix production and assayed for soluble β-glucan levels (106). As determined by in vitro biofilm assays, *IFD4* and *IFD6* have positive roles in matrix production, while *GCA1*, *GCA2*, *ADH4*, and *ADH5* have negative roles. Furthermore, chromatin immunoprecipitation experiments determined that Zap1 is a direct activator of *IFD4* and *IFD6* and an indirect repressor of *ADH4*, *ADH5*, *GCA1*, and *GCA2* (106). While these experiments have identified genes targeted by Zap1, the mechanism by which Zap1 mediates matrix formation within the biofilm is still unknown. One hypothesis is that a decrease in the concentration of zinc activates Zap1, which then regulates a set of genes resulting in a decrease in the production of extracellular matrix within the biofilm. Under low-flow conditions, zinc depletion may occur, resulting in a biofilm with little matrix (8). Under high-flow conditions, zinc would be replenished, resulting in an increase of biofilm matrix levels (8). The increased matrix may serve to strengthen the structural integrity of the biofilm during conditions of increased stress on the structure of the biofilm. Zap1 regulates the production of matrix through the induction or repression of genes. Zap1 inhibits the extracellular glucoamylases Gca1 and Gca2, which convert long-chain polysaccharides into smaller-chain polysaccharides (106). A likely role for Gca1 and Gca2 in matrix production is the hydrolytic release of soluble β-1,3 glucan fragments. There are many hypotheses for the mechanistic roles of the alcohol dehydrogenase-related gene products Ifd4, Ifd6, and Adh5. They may govern intermediary carbon metabolism to create energy for glucan synthesis, but this is complicated by the fact that Adh5 stimulates matrix production, while Ifd4 and Ifd6 inhibit matrix production. A second hypothesis is based on the roles of alcohol dehydrogenases in the Ehrlich pathway (50). This pathway allows nitrogen assimilation from amino

acids, yielding α-keto acids that must be reduced to acyl alcohols and aryl-alcohols for secretion. These alcohols have major roles in quorum sensing and cell signaling, where they can, among other things, either stimulate or inhibit hyphal growth (2, 23, 52, 90, 110, 121). A simple model can be imagined where Ifd4, Ifd6, and Adh5 catalyze the final reductive step in the biogenesis of biofilm-associated acyl alcohols and aryl-alcohols, with these alcohols acting as signals to control matrix synthesis. The opposing results of these gene products on matrix production may be related to substrate specificity. Thus, Ifd4 and Ifd6 may act preferentially to yield a matrix-inhibitory signal, while Adh5 may act preferentially to yield a matrix-stimulatory signal (106).

While many proteins influence the formation and maintenance of the extracellular matrix, other components also regulate the matrix, such as exopolysaccharides and extracellular DNA. In bacteria, exopolysaccharides are structural components of biofilms, aiding in adhesion and protecting the underlying cells from changes in the surrounding environment (5). In *C. albicans*, after 48 h of growth a colorless matrix of exopolysaccharides can be observed embedding the biofilm. The exopolysaccharides are composed of glucose, mannose, rhamnose, and *N*-acetylglucosamine. *C. albicans* produces an exopolysaccharide with a molecular mass of ~300 kDa, larger than most proteins produced by the cell (75). The exopolysaccharide provides a solid mesh around the cells of the *C. albicans* biofilm, providing structural support and protecting the biofilm from possible damage. Another nonprotein component of the extracellular matrix is extracellular DNA (eDNA). eDNA can be extracted from the extracellular matrix of a 72-h biofilm (114). The presence and importance of eDNA in biofilms are seen by the addition of DNase to a mature biofilm resulting in a 30% decrease of biofilm biomass and minor detachment of the biofilm (3, 91). The addition of eDNA at the beginning of biofilm development results in mature biofilms with increased biofilm biomass. eDNA levels in the extracellular matrix are affected by the nutrient conditions of the biofilm, with increased amounts of eDNA found in vitro in biofilms grown in filamentation media (91). This evidence indicates that eDNA is an important part of the matrix and contributes to the maintenance, structure, and stability of a mature biofilm.

CELL-CELL COMMUNICATION WITHIN A BIOFILM: QUORUM SENSING

Although bacteria and many fungi exist as single cells, they are able to coordinate with their neighboring cells their activities by secreting signaling factors and responding to these signals. Quorum sensing describes a broad set of phenomena where cell density-dependent responses are orchestrated through signaling by such secreted molecules (59). Quorum sensing governs functions as diverse as bioluminescence and virulence. It has a vital role in bacterial biofilm dynamics, and its role in *C. albicans* biofilms is now beginning to be understood.

In *C. albicans*, there are two main characterized quorum-sensing molecules, tyrosol and farnesol. Both compounds are dependent on the availability of amino acid precursors and are regulated by pH, oxygen availability, and nitrogen repression by ammonium (40). Both molecules are found under planktonic and biofilm conditions, and both are found to increase with biofilm maturation. The quorum-sensing factor tyrosol was originally identified in growth media of planktonic cells. Addition of tyrosol to yeast form cells accelerates growth and under hypha-inducing conditions can rapidly increase the formation of hyphal cells (23). Examination of biofilm tyrosol levels found an increased concentration in biofilms compared to planktonic cells. While tyrosol is unable to counteract the inhibitory effects of farnesol, it is able to increase the rate of hypha formation in early biofilms (2). These data taken together indicate a positive role for tyrosol in biofilm formation. One attractive hypothesis is that when conditions are ideal for biofilm formation, tyrosol acts to stimulate the rapid formation of a biofilm (2). This hypothesis is based on experiments where the addition of tyrosol accelerates the production of hyphae at between 2 and 6 h of biofilm development. Tyrosol production peaks during the first 24 h of biofilm formation and then levels off, indicating that it is functional during early biofilm formation (2). It is possible that tyrosol acts to only stimulate the initiation of biofilm formation, after which it no longer functions in biofilm formation. The ability to accelerate the production of hyphae would aid in establishing the biofilm quicker, increasing the difficulty for the host defenses to be able to combat the biofilm.

The most extensively studied *C. albicans* quorum-sensing molecule is farnesol. Farnesol was originally identified as a component in high-density cultures that inhibits hypha formation (52). Further studies indicated that it inhibits in vitro biofilm formation as well, perhaps as a consequence of hypha inhibition (121). While farnesol inhibits the formation of hyphal cells, it does not inhibit the elongation of established germ tubes (95). Farnesol is produced from an ergosterol biosynthetic intermediate, and while the precise mechanism by which it blocks hypha and biofilm formation is unclear, two recent studies shed light on potential downstream events (53). First, microarray analysis of farnesol-treated biofilms indicates that it controls expression of genes important for hyphal development (*TUP1* and *CRK1*), drug resistance (*FCR1* and *PDR16*), and cell wall integrity (*CHT2* and *CHT3*) (20). These data indicate a strong role for farnesol in preventing the development of hyphal cells by downregulating genes required for hyphal development and cell wall integrity. Second, mutant studies suggest that the histidine kinase Chk1, a member of a two-component signal transduction pathway, may be part of the farnesol signaling pathway (67). Unlike a wild-type strain, a *chk1*Δ/*chk1*Δ mutant strain will form a biofilm in the presence of farnesol, suggesting that this kinase is a key downstream mediator of the cellular response to quorum sensing (67). An interesting area for future study is to determine how the presence of farnesol is transmitted to Chk1 and to determine which aspects of the farnesol transcriptional response may be dependent upon Chk1 (20, 67). Since farnesol inhibits hypha formation and reduces biofilm biomass, the role for farnesol may be to stall or inhibit mature biofilm formation.

Why might microbes produce quorum-sensing factors to inhibit biofilm formation? One simple hypothesis is that the process aids in dispersal when nutrients are depleted by the mature biofilm, with the production and release of less adherent daughter cells allowing the population of remote surfaces (45, 46). *C. albicans* yeast cells, in general, exhibit less cell-cell adherence than hyphal cells. The dispersing cells envisioned in the quorum-sensing model (Fig. 2D) would need to be less adherent than the normal yeast cell. An interesting possibility is that yeast form cells may have mechanisms to regulate the activity of their adhesins.

In vivo, *C. albicans* is not the only organism seeking to create a biofilm or infect a host. This competition ranges

from fellow fungi to bacteria. While there are cases where fungi and bacteria form a symbiotic biofilm (1), often the different organisms become predatory to or parasitic of each other. While quorum-sensing molecules can function to control biofilm formation of *C. albicans*, evidence exists that they may also function to communicate with other species in its environment (149). Interestingly, quorum-sensing molecules released by *C. albicans* have been shown to negatively affect their neighbors. For instance, farnesol has strong antifungal activities against *Aspergillus nidulans* and *S. cerevisiae* (31). Farnesol also inhibits biofilm formation and lipase activity of *Staphylococcus aureus* while enhancing the antibiotic susceptibility of this pathogen (58, 72). Quorum-sensing molecules produced by *C. albicans* can be seen as bifunctional, as they affect both *C. albicans* and the bacteria that it comes into contact with in vivo. In one role they act to either stimulate rapid biofilm formation (tyrosol) or suppress biofilm formation through the inhibition of hypha formation (farnesol). In the second role one of the molecules, farnesol, acts to suppress growth and biofilm formation of a number of bacterial competitors. This activity would help to confer a selective advantage for *C. albicans* in their environment. These two roles may allow for *C. albicans* to form biofilms under the best nutrient conditions and to deter any possible competitors for competing for the location (20, 31, 40).

C. ALBICANS MATING PATHWAY

The mating pathway in *C. albicans* is a complex network of signaling and changes in cell morphology that does not involve meiosis. It is believed to occur rarely, with most clinical isolates being **a**/α and unable to mate, as only **a**/**a** or α/α cells are capable of mating. In addition to the requirement of being **a**/**a** or α/α, a morphological change needs to occur from the mating-incompetent white phenotype to the mating-competent opaque phenotype (87, 93). If mating does occur, a tetraploid is created which then loses chromosomes at random to produce a new diploid cell (56, 88). While the mating process is discussed in further detail in a previous chapter, here we examine its role in biofilm formation.

In nature, *C. albicans* populations are largely clonal, and while white-opaque switching occurs at a relatively low frequency, opaque cells of opposite mating type from different strains would be expected to encounter one another very rarely in nature to allow mating and genetic exchange. Even so, there is now evidence that the mating pathway may affect biofilm formation in some situations (12, 43). A recent study shows that the mating factors can improve biofilm formation by responsive cells, even if they are in the mating-incompetent white phase. This increase in biofilm formation is linked to an increase of cell adhesion in **a**/**a** cells when exposed to α-pheromone (26). In fact, the response of the white-phase cells to the mating pheromone is to trigger biofilm formation. This study also provides experimental support for the hypothesis that a biofilm is a mating-permissive environment, even for widely separated mating partners. In addition to the mating factor response, a large number of genes induced during filamentation are similarly induced during mating (150). One of these genes, the *HWP1* adhesin gene, is induced by pheromone (13, 150). While *HWP1* was initially reported to be induced only in the mating-incompetent white-phase cells (26), recent work indicates that it has a similar biofilm defect whether in the mating-competent opaque or the mating-incompetent white cells (35). The induction of adhesins and genes functioning in filamentation may explain how the induction of the mating response facilitates biofilm formation. *CZF1* also functions in both biofilm formation and mating. Czf1 controls contact-dependent filamentation, and a *czf1*Δ/*czf1*Δ strain results in biofilms with reduced mass (137). Interestingly, Czf1 regulates the conversion of white-phase cells to opaque-phase cells, a change that is essential for mating to occur. The data beg the question of whether the cellular pathways of biofilm formation and the pathways of mating are connected and how Czf1 and Hwp1 expression can affect both processes (69, 143). The biofilm formation ability of *C. albicans* almost certainly did not evolve to promote catheter-based infections; might it have evolved to support rare mating instead? One suggestion is that it is possible that mating may occur within the biofilm itself and that the conditions set up by a biofilm facilitate the mating process (26, 132). This condition would occur rarely, as biofilm populations have been observed to be largely clonal (43). Constraint-specific environmental conditions have been shown to induce mass switching of white cells to the opaque phase in some strains, suggesting that in appropriate host niches, biofilms may facilitate mating (26, 123). While this mating may occur, it is undoubtedly a very rare event likely to occur in biofilms under very specific environmental conditions.

DRUG RESISTANCE WITHIN A BIOFILM

A major concern of medical importance is the extreme resistance of *C. albicans* biofilms to many of the widely used azole antifungal agents (33, 48, 70). Analysis of this resistance has identified multiple mechanisms contributing to drug resistance. These include alterations in the sterol biosynthesis pathway, overexpression of the *ERG11* gene, mutations in the *ERG11* gene, and overexpression of membrane transport proteins, which reduces intracellular drug accumulation (94). The precise and complex mechanism of drug resistance is discussed in greater detail in another chapter in this book, and thus we focus on drug resistance in biofilms.

It has been shown that as the biofilm matures, the more resistant it becomes to antifungal treatments. Several hypotheses have been generated to explain why *C. albicans* biofilms are much more resistant to antifungal drugs than planktonic cultures. One attractive idea is that extracellular matrix prohibits antifungal drugs from access to growing cells. Some have contended that this is unlikely since resistance emerges relatively quickly (6 h) after *C. albicans* surface adherence, which they contend is prior to the secretion of extracellular matrix (96, 121). Furthermore, *C. albicans* biofilms grown in the absence of medium flow produce less extracellular matrix but are as resistant to azole drugs as biofilms produced in flowing media (49). One recent paper tries to tackles the question of whether antifungal drugs can diffuse through a *C. albicans* biofilm. Al-Fattani et al. used a novel biofilm filtration model and found that antifungal drugs diffuse readily through mature *C. albicans* biofilms (4). The biofilms formed on the filters in this assay were not grown under conditions that are known to favor matrix formation, such as flow, so the results must be taken with that caveat in mind. One other caveat is that while the drug may be able to penetrate a mature biofilm, that does not mean that it can access all parts of the biofilm. Also, the amount of antifungal used in the experiments far exceeded that normally used in in vivo treatment (4). Interestingly, it was observed that mixing yeast cells with the bacterium *Staphylococcus epidermidis*, commonly found in mixed biofilms in

vivo, slows drug penetration, suggesting that drug resistance in the clinic may be more complicated than most in vitro investigations imply (4). As mixed biofilms are observed in the clinic, and there is a host of other factors governing in vivo biofilm formation, it may be that a more robust biofilm forms in vivo than that reproduced in vitro. This difference may contribute to the resistance of the biofilms to antifungals.

Another hypothesis, that drug resistance in biofilms is due to increased expression of drug efflux pumps, may explain the early acquisition of drug resistance. Mateus et al. used green fluorescent protein-tagged drug efflux pumps to demonstrate an upregulation of Cdr1 in adherent cells compared to planktonic cells (92). They also showed that mutations in Mdr1 and Cdr1 decrease the resistance of biofilms to fluconazole and that a double mutant of these pumps abolishes resistance of early biofilms to the drug (92). Previous findings by Mukherjee et al., obtained using a different azole resistance measurement method, indicated that Cdr1, but not Mdr1, governs resistance in early biofilms (96). Thus, drug efflux pumps do play a role in the drug resistance of early biofilms, although the specific pumps involved may depend upon the precise experimental conditions. While these pumps appear to regulate drug resistance, their expression is always elevated in forming biofilms regardless of the presence of antifungals, indicating that the resistance is a by-product of biofilm formation and not developed to resist antifungal agents (92, 96).

Drug resistance in mature biofilms appears to involve distinct mechanisms. Unlike in early biofilms, resistance in mature biofilms does not rely on any known antifungal efflux pumps (96, 119). It has been hypothesized that a change in membrane sterol composition during biofilm formation may explain drug resistance to amphotericin B and azoles, which target sterols and sterol biosynthesis (96). This hypothesis was suggested by microarray analysis performed on planktonic cells from two azole-resistant strains. The microarray identified changes in expression of genes with roles in amino acid metabolism, carbohydrate metabolism, cell stress, cell wall maintenance, small-molecule transport, and lipid, fatty acid, and sterol metabolism (125). Mutant analysis indicates that the mitogen-activated protein kinase Mkc1 is a major regulator of azole resistance in mature biofilms (69). Biochemical characterization of Mkc1 further indicates that it is activated upon contact with a variety of surfaces (69); thus, it is a very appealing candidate for a mediator of biofilm cell-specific phenotypes. The *mkc1*Δ/*mkc1*Δ mutant strain has a slightly altered biofilm structure, but most striking is that the mutant biofilms are entirely azole sensitive. Thus, the analysis of Mkc1 targets may lead directly to an understanding of this novel azole resistance mechanism.

While azole resistance in early biofilms is the result of the expression of multidrug resistance pumps, the precise mechanism or series of mechanisms for resistance in mature biofilms remains largely unknown. Current research has uncovered fungal persister cells that function in mature biofilm drug resistance (74). Persister cells were first identified in bacterial biofilms, where it was observed that a small fraction of cells were not killed under very high levels of antibiotics. Whereas the surviving cells were essentially invulnerable, the bulk of the biofilm was still sensitive to the antibiotics (18). These cells, called persister cells, that survived neither grow nor die, and they are believed to be responsible for the bacterial biofilm's resistance to antibiotics (61). While the other cells died, the persister cells were able to impart survival to the rest of the biofilm (18). In *C. albicans* biofilms, a subset of cells (<1%) is never killed when exposed to high levels of drugs (amphotericin B, chlorhexidine, and fluconazole), similar to the persister cell phenomenon seen in the bacterial biofilms (74). Due to a <10-fold killing of biofilm cells, caspofungin was excluded from this study. Also, addition of both amphotericin B and chlorhexidine had no effect on the persister cells, indicating that the cells have multidrug resistance. Interestingly, cell populations produced from persister cells had no increased drug resistance over that seen in wild-type cultures. The persister cells have no morphological difference from other cells and, unlike with bacterial cultures, are only observed in biofilms. The drug resistance observed in mature biofilms can be explained in part by the presence of persister cells. As the antifungals kill other cells within the biofilm, the persister cells are immune and are able to provide the biofilm with new cells. This immunity prevents the antifungals from being able to fully eradicate the biofilm, with the biofilm re-forming after the end of treatment (74).

The study of biofilms is primarily done in vitro, and while successful, it is often difficult to replicate the in vivo environment in vitro. In vivo studies of drug susceptibility in biofilms has shown that exposure to high levels of azoles, which are lethal to in vitro cells, does not affect cell viability in mature biofilms in vivo. In fact, cells recovered from in vivo biofilms had a 128-fold-increased resistance to azoles over planktonic cells (7). In vivo analysis has determined that changes in cell wall and secreted carbohydrates are associated with biofilm growth and contribute to biofilm drug resistance (102). Furthermore, biofilm growth results in cell wall changes that adapt to the constantly changing responses to environmental signals, including the addition of antifungals. The predominant change to these environmental signals is in the quantity of β-1,3 glucans. Biofilm cell walls have a significantly higher concentration of β-1,3 glucans than that found in planktonic cells. The antifungal ability of biofilms can be correlated with this increased β-1,3 glucan level, as the addition of glucans to a biofilm enhances the effective activity of the antifungals. This provides strong evidence that in vivo, β-1,3 glucan is a component that contributes to the antifungal resistance of biofilms (101, 102). In *C. albicans*, calcineurin is required for virulence and for resistance to azole antifungals. Calcineurin mutant strains are sensitive to fluconazole under both planktonic and biofilm conditions. While biofilms are resistant to fluconazole or calcineurin inhibitors, a combination of either inhibitor with fluconazole resulted in a biofilm with large sensitivity to antifungals. In biofilms this sensitivity is hypothesized to occur by fluconazole decreasing the sterol concentrations in the biofilm, thus allowing the calcineurin inhibitors to penetrate the cell and cause cell death (141).

CONCLUDING REMARKS

In the last few years, our understanding of how *C. albicans* forms biofilms has exploded. Gene regulatory networks are being established, and our knowledge of which genes affect biofilm formation is steadily growing. We have begun to associate gene expression with the specific steps in the gene regulatory networks of biofilm formation and to better define the molecular and cellular biology that occurs at each of these steps. As work continues and the quorum-sensing molecules are further defined, we will be able to understand what triggers the progression from one step of biofilm formation to the next. More confidence will be placed on in vitro

results now that they can be confirmed with established and reproducible in vivo models. While major challenges lie ahead to further refine the gene regulatory networks and correlate them to in vivo results, it is nonetheless an exciting time for the field of *C. albicans* biofilm formation.

REFERENCES

1. **Adam, B., G. S. Baillie, and L. J. Douglas.** 2002. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *J. Med. Microbiol.* **51:**344–349.
2. **Alem, M. A., M. D. Oteef, T. H. Flowers, and L. J. Douglas.** 2006. Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryot. Cell* **5:**1770–1779.
3. **Al-Fattani, M. A., and L. J. Douglas.** 2006. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J. Med. Microbiol.* **55:**999–1008.
4. **Al-Fattani, M. A., and L. J. Douglas.** 2004. Penetration of *Candida* biofilms by antifungal agents. *Antimicrob. Agents Chemother.* **48:**3291–3297.
5. **Allison, D. G., B. Ruiz, C. SanJose, A. Jaspe, and P. Gilbert.** 1998. Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. *FEMS Microbiol. Lett.* **167:**179–184.
6. **Almirante, B., D. Rodriguez, M. Cuenca-Estrella, M. Almela, F. Sanchez, J. Ayats, C. Alonso-Tarres, J. L. Rodriguez-Tudela, and A. Pahissa.** 2006. Epidemiology, risk factors, and prognosis of *Candida parapsilosis* bloodstream infections: case-control population-based surveillance study of patients in Barcelona, Spain, from 2002 to 2003. *J. Clin. Microbiol.* **44:**1681–1685.
7. **Andes, D., J. Nett, P. Oschel, R. Albrecht, K. Marchillo, and A. Pitula.** 2004. Development and characterization of an in vivo central venous catheter *Candida albicans* biofilm model. *Infect. Immun.* **72:**6023–6031.
8. **Baillie, G. S., and L. J. Douglas.** 2000. Matrix polymers of *Candida* biofilms and their possible role in biofilm resistance to antifungal agents. *J. Antimicrob. Chemother.* **46:** 397–403.
9. **Baillie, G. S., and L. J. Douglas.** 1999. Role of dimorphism in the development of *Candida albicans* biofilms. *J. Med. Microbiol.* **48:**671–679.
10. **Banerjee, M., D. S. Thompson, A. Lazzell, P. L. Carlisle, C. Pierce, C. Monteagudo, J. L. Lopez-Ribot, and D. Kadosh.** 2008. UME6, a novel filament-specific regulator of *Candida albicans* hyphal extension and virulence. *Mol. Biol. Cell* **19:**1354–1365.
11. **Bates, S., D. M. MacCallum, G. Bertram, C. A. Munro, H. B. Hughes, E. T. Buurman, A. J. Brown, F. C. Odds, and N. A. Gow.** 2005. *Candida albicans* Pmr1p, a secretory pathway P-type Ca^{2+}/Mn^{2+}-ATPase, is required for glycosylation and virulence. *J. Biol. Chem.* **280:**23408–23415.
12. **Bennett, R. J., and A. D. Johnson.** 2005. Mating in *Candida albicans* and the search for a sexual cycle. *Annu. Rev. Microbiol.* **59:**233–255.
13. **Bennett, R. J., M. A. Uhl, M. G. Miller, and A. D. Johnson.** 2003. Identification and characterization of a *Candida albicans* mating pheromone. *Mol. Cell. Biol.* **23:**8189–8201.
14. **Berger, A.** 2002. What does zinc do? *BMJ* **325:**1062.
15. **Bird, A., M. V. Evans-Galea, E. Blankman, H. Zhao, H. Luo, D. R. Winge, and D. J. Eide.** 2000. Mapping the DNA binding domain of the Zap1 zinc-responsive transcriptional activator. *J. Biol. Chem.* **275:**16160–16166.
16. Reference deleted.
17. **Blankenship, J. R., S. Fanning, J. J. Hamaker, and A. P. Mitchell.** 2010. An extensive circuitry for cell wall regulation in *Candida albicans*. *PLoS Pathog.* **6:**e1000752.
18. **Brooun, A., S. Liu, and K. Lewis.** 2000. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob. Agents Chemother.* **44:**640–646.
19. **Brown, D. H., Jr., A. D. Giusani, X. Chen, and C. A. Kumamoto.** 1999. Filamentous growth of *Candida albicans* in response to physical environmental cues and its regulation by the unique CZF1 gene. *Mol. Microbiol.* **34:**651–662.
20. **Cao, Y. Y., Y. B. Cao, Z. Xu, K. Ying, Y. Li, Y. Xie, Z. Y. Zhu, W. S. Chen, and Y. Y. Jiang.** 2005. cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol. *Antimicrob. Agents Chemother.* **49:**584–589.
21. **Chaffin, W. L.** 2008. *Candida albicans* cell wall proteins. *Microbiol. Mol. Biol. Rev.* **72:**495–544.
22. **Chandra, J., D. M. Kuhn, P. K. Mukherjee, L. L. Hoyer, T. McCormick, and M. A. Ghannoum.** 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J. Bacteriol.* **183:**5385–5394.
23. **Chen, H., M. Fujita, Q. Feng, J. Clardy, and G. R. Fink.** 2004. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **101:**5048–5052.
24. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. *Science* **284:**1318–1322.
25. **Crowe, J. D., I. K. Sievwright, G. C. Auld, N. R. Moore, N. A. Gow, and N. A. Booth.** 2003. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. *Mol. Microbiol.* **47:**1637–1651.
26. **Daniels, K. J., T. Srikantha, S. R. Lockhart, C. Pujol, and D. R. Soll.** 2006. Opaque cells signal white cells to form biofilms in *Candida albicans*. *EMBO J.* **25:**2240–2252.
27. **Davis, D., R. B. Wilson, and A. P. Mitchell.** 2000. RIM101-dependent and -independent pathways govern pH responses in *Candida albicans*. *Mol. Cell. Biol.* **20:** 971–978.
28. **Davis, D. A., V. M. Bruno, L. Loza, S. G. Filler, and A. P. Mitchell.** 2002. *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. *Genetics* **162:**1573–1581.
29. **Davis, S. C., C. Ricotti, A. Cazzaniga, E. Welsh, W. H. Eaglstein, and P. M. Mertz.** 2008. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. *Wound Repair Regen.* **16:**23–29.
30. **Davis-Hanna, A., A. E. Piispanen, L. I. Stateva, and D. A. Hogan.** 2008. Farnesol and dodecanol effects on the *Candida albicans* Ras1-cAMP signalling pathway and the regulation of morphogenesis. *Mol. Microbiol.* **67:**47–62.
31. **De Sordi, L., and F. A. Muhlschlegel.** 2009. Quorum sensing and fungal-bacterial interactions in *Candida albicans*: a communicative network regulating microbial coexistence and virulence. *FEMS Yeast Res.* **9:**990–999.
32. **Ding, C., and G. Butler.** 2007. Development of a gene knockout system in *Candida parapsilosis* reveals a conserved role for BCR1 in biofilm formation. *Eukaryot. Cell* **6:**1310–1319.
33. **Douglas, L. J.** 2003. *Candida* biofilms and their role in infection. *Trends Microbiol.* **11:**30–36.
34. **Dranginis, A. M., J. M. Rauceo, J. E. Coronado, and P. N. Lipke.** 2007. A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. *Microbiol. Mol. Biol. Rev.* **71:**282–294.
35. **Ene, I. V., and R. J. Bennett.** 2009. Hwp1 and related adhesins contribute to both mating and biofilm formation in *Candida albicans*. *Eukaryot. Cell* **8:**1909–1913.
36. **Forrest, G. N., E. Weekes, and J. K. Johnson.** 2008. Increasing incidence of *Candida parapsilosis* candidemia with caspofungin usage. *J. Infect.* **56:**126–129.

37. **Fridkin, S. K., D. Kaufman, J. R. Edwards, S. Shetty, and T. Horan.** 2006. Changing incidence of *Candida* bloodstream infections among NICU patients in the United States: 1995–2004. *Pediatrics* **117:**1680–1687.
38. **Garcia-Effron, G., S. K. Katiyar, S. Park, T. D. Edlind, and D. S. Perlin.** 2008. A naturally occurring proline-to-alanine amino acid change in Fks1p in *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* accounts for reduced echinocandin susceptibility. *Antimicrob. Agents Chemother.* **52:**2305–2312.
39. **Garcia-Sanchez, S., S. Aubert, I. Iraqui, G. Janbon, J. M. Ghigo, and C. d'Enfert.** 2004. *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. *Eukaryot. Cell* **3:**536–545.
40. **Ghosh, S., B. W. Kebaara, A. L. Atkin, and K. W. Nickerson.** 2008. Regulation of aromatic alcohol production in *Candida albicans*. *Appl. Environ. Microbiol.* **74:**7211–7218.
41. **Goyard, S., P. Knechtle, M. Chauvel, A. Mallet, M. C. Prevost, C. Proux, J. Y. Coppee, P. Schwartz, F. Dromer, H. Park, S. G. Filler, G. Janbon, and C. d'Enfert.** 2008. The Yak1 kinase is involved in the initiation and maintenance of hyphal growth in *Candida albicans*. *Mol. Biol. Cell* **19:**2251–2266.
42. **Granger, B. L., M. L. Flenniken, D. A. Davis, A. P. Mitchell, and J. E. Cutler.** 2005. Yeast wall protein 1 of *Candida albicans*. *Microbiology* **151:**1631–1644.
43. **Graser, Y., M. Volovsek, J. Arrington, G. Schonian, W. Presber, T. G. Mitchell, and R. Vilgalys.** 1996. Molecular markers reveal that population structure of the human pathogen *Candida albicans* exhibits both clonality and recombination. *Proc. Natl. Acad. Sci. USA* **93:**12473–12477.
44. **Green, C. B., G. Cheng, J. Chandra, P. Mukherjee, M. A. Ghannoum, and L. L. Hoyer.** 2004. RT-PCR detection of *Candida albicans ALS* gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. *Microbiology* **150:**267–275.
45. **Greenberg, E. P.** 2003. Bacterial communication and group behavior. *J. Clin. Investig.* **112:**1288–1290.
46. **Greenberg, E. P.** 2003. Bacterial communication: tiny teamwork. *Nature* **424:**134.
47. **Hajjeh, R. A., A. N. Sofair, L. H. Harrison, G. M. Lyon, B. A. Arthington-Skaggs, S. A. Mirza, M. Phelan, J. Morgan, W. Lee-Yang, M. A. Ciblak, L. E. Benjamin, L. T. Sanza, S. Huie, S. F. Yeo, M. E. Brandt, and D. W. Warnock.** 2004. Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J. Clin. Microbiol.* **42:**1519–1527.
48. **Hawser, S.** 1996. Comparisons of the susceptibilities of planktonic and adherent *Candida albicans* to antifungal agents: a modified XTT tetrazolium assay using synchronised *C. albicans* cells. *J. Med. Vet. Mycol.* **34:**149–152.
49. **Hawser, S. P., G. S. Baillie, and L. J. Douglas.** 1998. Production of extracellular matrix by *Candida albicans* biofilms. *J. Med. Microbiol.* **47:**253–256.
50. **Hazelwood, L. A., J. M. Daran, A. J. van Maris, J. T. Pronk, and J. R. Dickinson.** 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Appl. Environ. Microbiol.* **74:**2259–2266.
51. **Hiller, E., S. Heine, H. Brunner, and S. Rupp.** 2007. *Candida albicans* Sun41p, a putative glycosidase, is involved in morphogenesis, cell wall biogenesis, and biofilm formation. *Eukaryot. Cell* **6:**2056–2065.
52. **Hornby, J. M., E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, and K. W. Nickerson.** 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol.* **67:**2982–2992.
53. **Hornby, J. M., B. W. Kebaara, and K. W. Nickerson.** 2003. Farnesol biosynthesis in *Candida albicans*: cellular response to sterol inhibition by zaragozic acid B. *Antimicrob. Agents Chemother.* **47:**2366–2369.
54. **Hoyer, L. L.** 2001. The *ALS* gene family of *Candida albicans*. *Trends Microbiol.* **9:**176–180.
55. **Hoyer, L. L., T. L. Payne, M. Bell, A. M. Myers, and S. Scherer.** 1998. *Candida albicans ALS3* and insights into the nature of the *ALS* gene family. *Curr. Genet.* **33:**451–459.
56. **Hull, C. M., R. M. Raisner, and A. D. Johnson.** 2000. Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. *Science* **289:**307–310.
57. **Imamura, Y., J. Chandra, P. K. Mukherjee, A. A. Lattif, L. B. Szczotka-Flynn, E. Pearlman, J. H. Lass, K. O'Donnell, and M. A. Ghannoum.** 2008. *Fusarium* and *Candida albicans* biofilms on soft contact lenses: model development, influence of lens type, and susceptibility to lens care solutions. *Antimicrob. Agents Chemother.* **52:**171–182.
58. **Jabra-Rizk, M. A., T. F. Meiller, C. E. James, and M. E. Shirtliff.** 2006. Effect of farnesol on *Staphylococcus aureus* biofilm formation and antimicrobial susceptibility. *Antimicrob. Agents Chemother.* **50:**1463–1469.
59. **Keller, L., and M. G. Surette.** 2006. Communication in bacteria: an ecological and evolutionary perspective. *Nat. Rev. Microbiol.* **4:**249–258.
60. **Kelly, M. T., D. M. MacCallum, S. D. Clancy, F. C. Odds, A. J. Brown, and G. Butler.** 2004. The *Candida albicans* CaACE2 gene affects morphogenesis, adherence and virulence. *Mol. Microbiol.* **53:**969–983.
61. **Keren, I., N. Kaldalu, A. Spoering, Y. Wang, and K. Lewis.** 2004. Persister cells and tolerance to antimicrobials. *FEMS Microbiol. Lett.* **230:**13–18.
62. **Kim, M. J., M. Kil, J. H. Jung, and J. Kim.** 2008. Roles of zinc-responsive transcription factor Csr1 in filamentous growth of the pathogenic yeast *Candida albicans*. *J. Microbiol. Biotechnol.* **18:**242–247.
63. **Kim, W. I., W. B. Lee, K. Song, and J. Kim.** 2000. Identification of a putative DEAD-box RNA helicase and a zinc-finger protein in *Candida albicans* by functional complementation of the *S. cerevisiae rok1* mutation. *Yeast* **16:**401–409.
64. **Klotz, S. A., N. K. Gaur, R. De Armond, D. Sheppard, N. Khardori, J. E. Edwards, Jr., P. N. Lipke, and M. El-Azizi.** 2007. *Candida albicans* Als proteins mediate aggregation with bacteria and yeasts. *Med. Mycol.* **45:**363–370.
65. **Klotz, S. A., M. L. Pendrak, and R. C. Hein.** 2001. Antibodies to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins react with *Candida albicans* alcohol dehydrogenase. *Microbiology* **147:**3159–3164.
66. **Kojic, E. M., and R. O. Darouiche.** 2004. *Candida* infections of medical devices. *Clin. Microbiol. Rev.* **17:**255–267.
67. **Kruppa, M., B. P. Krom, N. Chauhan, A. V. Bambach, R. L. Cihlar, and R. A. Calderone.** 2004. The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. *Eukaryot. Cell* **3:**1062–1065.
68. **Kulkarni, R. D., H. S. Kelkar, and R. A. Dean.** 2003. An eight-cysteine-containing CFEM domain unique to a group of fungal membrane proteins. *Trends Biochem. Sci.* **28:**118–121.
69. **Kumamoto, C. A.** 2005. A contact-activated kinase signals *Candida albicans* invasive growth and biofilm development. *Proc. Natl. Acad. Sci. USA* **102:**5576–5581.
70. **Kumamoto, C. A.** 2002. *Candida* biofilms. *Curr. Opin. Microbiol.* **5:**608–611.
71. **Kumamoto, C. A., and M. D. Vinces.** 2005. Alternative *Candida albicans* lifestyles: growth on surfaces. *Annu. Rev. Microbiol.* **59:**113–133.
72. **Kuroda, M., S. Nagasaki, R. Ito, and T. Ohta.** 2007. Sesquiterpene farnesol as a competitive inhibitor of lipase ac-

tivity of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **273:**28–34.
73. **Laffey, S. F., and G. Butler.** 2005. Phenotype switching affects biofilm formation by *Candida parapsilosis*. *Microbiology* **151:**1073–1081.
74. **LaFleur, M. D., C. A. Kumamoto, and K. Lewis.** 2006. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob. Agents Chemother.* **50:**3839–3846.
75. **Lal, P., D. Sharma, P. Pruthi, and V. Pruthi.** 2010. Exopolysaccharide analysis of biofilm-forming *Candida albicans*. *J. Appl. Microbiol.* **109:**128–136.
76. **Lamarre, C., N. Deslauriers, and Y. Bourbonnais.** 2000. Expression cloning of the *Candida albicans CSA1* gene encoding a mycelial surface antigen by sorting of *Saccharomyces cerevisiae* transformants with monoclonal antibody-coated magnetic beads. *Mol. Microbiol.* **35:**444–453.
77. **Lane, S., C. Birse, S. Zhou, R. Matson, and H. Liu.** 2001. DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in *Candida albicans*. *J. Biol. Chem.* **276:**48988–48996.
78. **Lane, S., S. Zhou, T. Pan, Q. Dai, and H. Liu.** 2001. The basic helix-loop-helix transcription factor Cph2 regulates hyphal development in *Candida albicans* partly via Tec1. *Mol. Cell. Biol.* **21:**6418–6428.
79. **Levin, A. S., S. F. Costa, N. S. Mussi, M. Basso, S. I. Sinto, C. Machado, D. C. Geiger, M. C. Villares, A. Z. Schreiber, A. A. Barone, and M. L. Branchini.** 1998. *Candida parapsilosis* fungemia associated with implantable and semi-implantable central venous catheters and the hands of healthcare workers. *Diagn. Microbiol. Infect. Dis.* **30:**243–249.
80. **Levy, I., L. G. Rubin, S. Vasishtha, V. Tucci, and S. K. Sood.** 1998. Emergence of *Candida parapsilosis* as the predominant species causing candidemia in children. *Clin. Infect. Dis.* **26:**1086–1088.
81. **Lewis, K.** 2001. Riddle of biofilm resistance. *Antimicrob. Agents Chemother.* **45:**999–1007.
82. **Lewis, R. E., H. J. Lo, I. I. Raad, and D. P. Kontoyiannis.** 2002. Lack of catheter infection by the *efg1/efg1 cph1/cph1* double-null mutant, a *Candida albicans* strain that is defective in filamentous growth. *Antimicrob. Agents Chemother.* **46:**1153–1155.
83. **Li, F., and S. P. Palecek.** 2008. Distinct domains of the *Candida albicans* adhesin Eap1p mediate cell-cell and cell-substrate interactions. *Microbiology* **154:**1193–1203.
84. **Li, F., and S. P. Palecek.** 2003. *EAP1*, a *Candida albicans* gene involved in binding human epithelial cells. *Eukaryot. Cell* **2:**1266–1273.
85. **Li, F., M. J. Svarovsky, A. J. Karlsson, J. P. Wagner, K. Marchillo, P. Oshel, D. Andes, and S. P. Palecek.** 2007. Eap1p, an adhesin that mediates *Candida albicans* biofilm formation in vitro and in vivo. *Eukaryot. Cell* **6:**931–939.
86. **Lochhead, P. A., G. Sibbet, N. Morrice, and V. Cleghon.** 2005. Activation-loop autophosphorylation is mediated by a novel transitional intermediate form of DYRKs. *Cell* **121:**925–936.
87. **Lockhart, S. R., R. Zhao, K. J. Daniels, and D. R. Soll.** 2003. Alpha-pheromone-induced "shmooing" and gene regulation require white-opaque switching during *Candida albicans* mating. *Eukaryot. Cell* **2:**847–855.
88. **Magee, B. B., and P. T. Magee.** 2000. Induction of mating in *Candida albicans* by construction of MTLa and MTLα strains. *Science* **289:**310–313.
89. **Marrie, T. J., and J. W. Costerton.** 1984. Scanning and transmission electron microscopy of in situ bacterial colonization of intravenous and intraarterial catheters. *J. Clin. Microbiol.* **19:**687–693.
90. **Martins, M., M. Henriques, J. Azeredo, S. M. Rocha, M. A. Coimbra, and R. Oliveira.** 2007. Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells. *Eukaryot. Cell* **6:**2429–2436.
91. **Martins, M., P. Uppuluri, D. P. Thomas, I. A. Cleary, M. Henriques, J. L. Lopez-Ribot, and R. Oliveira.** 2010. Presence of extracellular DNA in the *Candida albicans* biofilm matrix and its contribution to biofilms. *Mycopathologia* **169:**323–331.
92. **Mateus, C., S. A. Crow, Jr., and D. G. Ahearn.** 2004. Adherence of *Candida albicans* to silicone induces immediate enhanced tolerance to fluconazole. *Antimicrob. Agents Chemother.* **48:**3358–3366.
93. **Miller, M. G., and A. D. Johnson.** 2002. White-opaque switching in *Candida albicans* is controlled by mating-type locus homeodomain proteins and allows efficient mating. *Cell* **110:**293–302.
94. **Morschhauser, J.** 2002. The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochim. Biophys. Acta* **1587:**240–248.
95. **Mosel, D. D., R. Dumitru, J. M. Hornby, A. L. Atkin, and K. W. Nickerson.** 2005. Farnesol concentrations required to block germ tube formation in *Candida albicans* in the presence and absence of serum. *Appl. Environ. Microbiol.* **71:**4938–4940.
96. **Mukherjee, P. K., J. Chandra, D. M. Kuhn, and M. A. Ghannoum.** 2003. Mechanism of fluconazole resistance in *Candida albicans* biofilms: phase-specific role of efflux pumps and membrane sterols. *Infect. Immun.* **71:**4333–4340.
97. **Mukherjee, P. K., S. Mohamed, J. Chandra, D. Kuhn, S. Liu, O. S. Antar, R. Munyon, A. P. Mitchell, D. Andes, M. R. Chance, M. Rouabhia, and M. A. Ghannoum.** 2006. Alcohol dehydrogenase restricts the ability of the pathogen *Candida albicans* to form a biofilm on catheter surfaces through an ethanol-based mechanism. *Infect. Immun.* **74:**3804–3816.
98. **Munro, C. A., D. A. Schofield, G. W. Gooday, and N. A. Gow.** 1998. Regulation of chitin synthesis during dimorphic growth of *Candida albicans*. *Microbiology* **144**(Pt. 2)**:**391–401.
99. **Murillo, L. A., G. Newport, C. Y. Lan, S. Habelitz, J. Dungan, and N. M. Agabian.** 2005. Genome-wide transcription profiling of the early phase of biofilm formation by *Candida albicans*. *Eukaryot. Cell* **4:**1562–1573.
100. **Nantel, A., D. Dignard, C. Bachewich, D. Harcus, A. Marcil, A. P. Bouin, C. W. Sensen, H. Hogues, M. van het Hoog, P. Gordon, T. Rigby, F. Benoit, D. C. Tessier, D. Y. Thomas, and M. Whiteway.** 2002. Transcription profiling of *Candida albicans* cells undergoing the yeast-to-hyphal transition. *Mol. Biol. Cell* **13:**3452–3465.
101. **Nett, J., L. Lincoln, K. Marchillo, and D. Andes.** 2007. Beta-1,3 glucan as a test for central venous catheter biofilm infection. *J. Infect. Dis.* **195:**1705–1712.
102. **Nett, J., L. Lincoln, K. Marchillo, R. Massey, K. Holoyda, B. Hoff, M. VanHandel, and D. Andes.** 2007. Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob. Agents Chemother.* **51:** 510–520.
103. **Nobile, C. J., D. R. Andes, J. E. Nett, F. J. Smith, F. Yue, Q. T. Phan, J. E. Edwards, S. G. Filler, and A. P. Mitchell.** 2006. Critical role of Bcr1-dependent adhesins in *C. albicans* biofilm formation in vitro and in vivo. *PLoS Pathog.* **2:**e63.
104. **Nobile, C. J., and A. P. Mitchell.** 2005. Regulation of cell-surface genes and biofilm formation by the *C. albicans* transcription factor Bcr1p. *Curr. Biol.* **15:**1150–1155.
105. **Nobile, C. J., J. E. Nett, D. R. Andes, and A. P. Mitchell.** 2006. Function of *Candida albicans* adhesin Hwp1 in biofilm formation. *Eukaryot. Cell* **5:**1604–1610.

106. **Nobile, C. J., J. E. Nett, A. D. Hernday, O. R. Homann, J.-S. Deneault, A. Nantel, D. R. Andes, A. D. Johnson, and A. P. Mitchell.** 2009. Biofilm matrix regulation by *Candida albicans* Zap1. *PLoS Biol.* **7:**1000133.
107. **Nobile, C. J., H. A. Schneider, J. E. Nett, D. C. Sheppard, S. G. Filler, D. R. Andes, and A. P. Mitchell.** 2008. Complementary adhesin function in C. *albicans* biofilm formation. *Curr. Biol.* **18:**1017–1024.
108. **Norice, C. T., F. J. Smith, Jr., N. Solis, S. G. Filler, and A. P. Mitchell.** 2007. Requirement for *Candida albicans* Sun41 in biofilm formation and virulence. *Eukaryot. Cell* **6:**2046–2055.
109. **O'Connor, L., S. Lahiff, F. Casey, M. Glennon, M. Cormican, and M. Maher.** 2005. Quantification of *ALS1* gene expression in *Candida albicans* biofilms by RT-PCR using hybridisation probes on the LightCycler. *Mol. Cell. Probes* **19:**153–162.
110. **Oh, K. B., H. Miyazawa, T. Naito, and H. Matsuoka.** 2001. Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **98:**4664–4668.
111. **Otoo, H. N., K. G. Lee, W. Qiu, and P. N. Lipke.** 2008. *Candida albicans* Als adhesins have conserved amyloid-forming sequences. *Eukaryot. Cell* **7:**776–782.
112. **Palanisamy, S. K., M. A. Ramirez, M. Lorenz, and S. A. Lee.** 2010. *Candida albicans PEP12* is required for biofilm integrity and in vivo virulence. *Eukaryot. Cell* **9:**266–277.
113. **Pappas, P. G., J. H. Rex, J. D. Sobel, S. G. Filler, W. E. Dismukes, T. J. Walsh, and J. E. Edwards.** 2004. Guidelines for treatment of candidiasis. *Clin. Infect. Dis.* **38:**161–189.
114. **Paramonova, E., B. P. Krom, H. C. van der Mei, H. J. Busscher, and P. K. Sharma.** 2009. Hyphal content determines the compression strength of *Candida albicans* biofilms. *Microbiology* **155:**1997–2003.
115. **Parsek, M. R., and P. K. Singh.** 2003. Bacterial biofilms: an emerging link to disease pathogenesis. *Annu. Rev. Microbiol.* **57:**677–701.
116. **Peltroche-Llacsahuanga, H., S. Goyard, C. d'Enfert, S. K. Prill, and J. F. Ernst.** 2006. Protein *O*-mannosyltransferase isoforms regulate biofilm formation in *Candida albicans*. *Antimicrob. Agents Chemother.* **50:**3488–3491.
117. **Perez, A., B. Pedros, A. Murgui, M. Casanova, J. L. Lopez-Ribot, and J. P. Martinez.** 2006. Biofilm formation by *Candida albicans* mutants for genes coding fungal proteins exhibiting the eight-cysteine-containing CFEM domain. *FEMS Yeast Res.* **6:**1074–1084.
118. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
119. **Ramage, G., S. Bachmann, T. F. Patterson, B. L. Wickes, and J. L. Lopez-Ribot.** 2002. Investigation of multidrug efflux pumps in relation to fluconazole resistance in *Candida albicans* biofilms. *J. Antimicrob. Chemother.* **49:**973–980.
120. **Ramage, G., E. Mowat, B. Jones, C. Williams, and J. Lopez-Ribot.** 2009. Our current understanding of fungal biofilms. *Crit. Rev. Microbiol.* **35:**340–355.
121. **Ramage, G., S. P. Saville, B. L. Wickes, and J. L. Lopez-Ribot.** 2002. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl. Environ. Microbiol.* **68:**5459–5463.
122. **Ramage, G., K. VandeWalle, J. L. Lopez-Ribot, and B. L. Wickes.** 2002. The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol. Lett.* **214:**95–100.
123. **Ramirez-Zavala, B., O. Reuss, Y. N. Park, K. Ohlsen, and J. Morschhauser.** 2008. Environmental induction of white-opaque switching in *Candida albicans*. *PLoS Pathog.* **4:**e1000089.
124. **Richard, M. L., C. J. Nobile, V. M. Bruno, and A. P. Mitchell.** 2005. *Candida albicans* biofilm-defective mutants. *Eukaryot. Cell* **4:**1493–1502.
125. **Rogers, P. D., and K. S. Barker.** 2002. Evaluation of differential gene expression in fluconazole-susceptible and -resistant isolates of *Candida albicans* by cDNA microarray analysis. *Antimicrob. Agents Chemother.* **46:** 3412–3417.
126. **Schweizer, A., S. Rupp, B. N. Taylor, M. Rollinghoff, and K. Schroppel.** 2000. The TEA/ATTS transcription factor CaTec1p regulates hyphal development and virulence in *Candida albicans*. *Mol. Microbiol.* **38:**435–445.
127. **Setiadi, E. R., T. Doedt, F. Cottier, C. Noffz, and J. F. Ernst.** 2006. Transcriptional response of *Candida albicans* to hypoxia: linkage of oxygen sensing and Efg1p-regulatory networks. *J. Mol. Biol.* **361:**399–411.
128. **Shapiro, S., E. Giertsen, and B. Guggenheim.** 2002. An in vitro oral biofilm model for comparing the efficacy of antimicrobial mouthrinses. *Caries Res.* **36:**93–100.
129. **Sharkey, L. L., M. D. McNemar, S. M. Saporito-Irwin, P. S. Sypherd, and W. A. Fonzi.** 1999. HWP1 functions in the morphological development of *Candida albicans* downstream of EFG1, TUP1, and RBF1. *J. Bacteriol.* **181:**5273–5279.
130. **Sheppard, D. C., M. R. Yeaman, W. H. Welch, Q. T. Phan, Y. Fu, A. S. Ibrahim, S. G. Filler, M. Zhang, A. J. Waring, and J. E. Edwards, Jr.** 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. *J. Biol. Chem.* **279:**30480–30489.
131. **Sohn, K., C. Urban, H. Brunner, and S. Rupp.** 2003. EFG1 is a major regulator of cell wall dynamics in *Candida albicans* as revealed by DNA microarrays. *Mol. Microbiol.* **47:**89–102.
132. **Soll, D. R.** 2009. Why does *Candida albicans* switch? *FEMS Yeast Res.* **9:**973–989.
133. **Sonneborn, A., D. P. Bockmuhl, and J. F. Ernst.** 1999. Chlamydospore formation in *Candida albicans* requires the Efg1p morphogenetic regulator. *Infect. Immun.* **67:** 5514–5517.
134. **Sonneborn, A., B. Tebarth, and J. F. Ernst.** 1999. Control of white-opaque phenotypic switching in *Candida albicans* by the Efg1p morphogenetic regulator. *Infect. Immun.* **67:**4655–4660.
135. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283:**1535–1538.
136. **Staab, J. F., C. A. Ferrer, and P. Sundstrom.** 1996. Developmental expression of a tandemly repeated, proline-and glutamine-rich amino acid motif on hyphal surfaces on *Candida albicans*. *J. Biol. Chem.* **271:** 6298–6305.
137. **Stichternoth, C., and J. F. Ernst.** 2009. Hypoxic adaptation by Efg1 regulates biofilm formation by *Candida albicans*. *Appl. Environ. Microbiol.* **75:**3663–3672.
138. **Stoldt, V. R., A. Sonneborn, C. E. Leuker, and J. F. Ernst.** 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J.* **16:**1982–1991.
139. **Sundstrom, P., J. E. Cutler, and J. F. Staab.** 2002. Reevaluation of the role of *HWP1* in systemic candidiasis by use of *Candida albicans* strains with selectable marker *URA3* targeted to the *ENO1* locus. *Infect. Immun.* **70:**3281–3283.

140. **Tripathi, G., C. Wiltshire, S. Macaskill, H. Tournu, S. Budge, and A. J. Brown.** 2002. Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in *Candida albicans*. *EMBO J.* **21:**5448–5456.
141. **Uppuluri, P., J. Nett, J. Heitman, and D. Andes.** 2008. Synergistic effect of calcineurin inhibitors and fluconazole against *Candida albicans* biofilms. *Antimicrob. Agents Chemother.* **52:**1127–1132.
142. **Vinces, M. D., C. Haas, and C. A. Kumamoto.** 2006. Expression of the *Candida albicans* morphogenesis regulator gene *CZF1* and its regulation by Efg1p and Czf1p. *Eukaryot. Cell* **5:**825–835.
143. **Vinces, M. D., and C. A. Kumamoto.** 2007. The morphogenetic regulator Czf1p is a DNA-binding protein that regulates white opaque switching in *Candida albicans*. *Microbiology* **153:**2877–2884.
144. **Viudes, A., J. Peman, E. Canton, P. Ubeda, J. L. Lopez-Ribot, and M. Gobernado.** 2002. Candidemia at a tertiary-care hospital: epidemiology, treatment, clinical outcome and risk factors for death. *Eur. J. Clin. Microbiol. Infect. Dis.* **21:**767–774.
145. **Weissman, Z., and D. Kornitzer.** 2004. A family of *Candida* cell surface haem-binding proteins involved in haemin and haemoglobin-iron utilization. *Mol. Microbiol.* **53:**1209–1220.
146. **Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel.** 1988. Hospital-acquired candidemia. The attributable mortality and excess length of stay. *Arch. Intern. Med.* **148:**2642–2645.
147. **Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel.** 1989. Risk factors for hospital-acquired candidemia. A matched case-control study. *Arch. Intern. Med.* **149:**2349–2353.
148. **Wilson, L. S., C. M. Reyes, M. Stolpman, J. Speckman, K. Allen, and J. Beney.** 2002. The direct cost and incidence of systemic fungal infections. *Value Health* **5:**26–34.
149. **Xu, X. L., R. T. Lee, H. M. Fang, Y. M. Wang, R. Li, H. Zou, Y. Zhu, and Y. Wang.** 2008. Bacterial peptidoglycan triggers *Candida albicans* hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell Host Microbe* **4:**28–39.
150. **Zhao, R., K. J. Daniels, S. R. Lockhart, K. M. Yeater, L. L. Hoyer, and D. R. Soll.** 2005. Unique aspects of gene expression during *Candida albicans* mating and possible G_1 dependency. *Eukaryot. Cell* **4:**1175–1190.
151. **Zhao, X., K. J. Daniels, S. H. Oh, C. B. Green, K. M. Yeater, D. R. Soll, and L. L. Hoyer.** 2006. *Candida albicans* Als3p is required for wild-type biofilm formation on silicone elastomer surfaces. *Microbiology* **152:**2287–2299.
152. **Zhao, X., S. H. Oh, K. M. Yeater, and L. L. Hoyer.** 2005. Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. *Microbiology* **151:**1619–1630.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch20

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Candida spp. in Microbial Populations and Communities: Molecular Interactions and Biological Importance

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In almost every niche where *Candida* spp. are found within the host, diverse cocolonizing microbial species are often also present. The physical and chemical interactions between organisms likely have profound effects on health, yet we lack a deep understanding of the relationships within these polymicrobial communities and the relationships between polymicrobial communities and the host. *Candida*-bacterium interactions have been best studied for *Candida albicans*, and these interactions are the major focus of this chapter; the interactions between bacteria and other *Candida* species, as well as relationships between *C. albicans* and other fungal pathogens, are also described. This chapter emphasizes the underlying mechanisms that govern these interactions and their potential relevance to disease. As we gain an understanding of how microbes behave within polymicrobial communities, we now appreciate that *intraspecies* communication within microbial populations affects interspecies dynamics. Thus, we review cell-cell regulation within *Candida* single-species populations, with a focus on an important autoregulatory molecule, farnesol. The potential roles that farnesol plays in polymicrobial communities are discussed. By considering interspecies dynamics, we not only will gain information about ways that microbe-microbe interactions may reduce or enhance disease progression but also may uncover information that can translate into novel treatments or improved uses of currently existing therapies. Also, readers are directed to chapter 19 on biofilm formation.

POLYMICROBIAL BIOFILM INTERACTIONS ON ABIOTIC SURFACES AND EFFECTS ON RESISTANCE TO ANTIMICROBIALS

C. albicans, a fungus that generally lives as a commensal in healthy individuals, remains the most common cause of mycosis in humans with underlying medical issues, particularly those with indwelling medical devices. On implanted medical devices, such as intravenous or central venous catheters, endotracheal tubes, and medical prostheses, microorganisms, including *Candida* spp., predominantly exist in complex assemblages called biofilms, which are defined as cells aggregated on a surface through a combination of cell-cell interactions and an association with an extracellular matrix. Within a biofilm, cells assume characteristics not observed in planktonic cultures, such as increased resistance to stress, antimicrobials, and phagocytosis (39, 67).

Many biofilms within the host are not simply single-species assemblages, but rather, dynamic polymicrobial communities. *Candida* spp. in biofilms on medical devices are most often derived from the host's own endogenous flora (reviewed in reference 135), and other microflora organisms, such as staphylococci, are also often associated with catheters in both the presence and absence of *C. albicans* (67, 139). The gram-positive bacterial pathogen *Staphylococcus aureus*, which forms weak biofilms on abiotic surfaces in vitro unless specific surface treatments or growth conditions are provided (19), forms robust biofilms in the presence of *C. albicans* (70), in part through *S. aureus* association with *C. albicans* hyphae. The capacity to form biofilms with *C. albicans* may provide a means for *S. aureus* attachment and subsequent entry into the body. Moreover, the mixed-species biofilms are more resistant to vancomycin, and this additive effect appears to require a physical interaction, since when the two microbes are separated by a membrane, the effect is no longer observed. Intriguingly, in a mixed *S. aureus*-*C. albicans* biofilm, *S. aureus* cells appear to be coated with matrix components that are distinct from those observed in single species *S. aureus* biofilms (70), suggesting that the presence of *C. albicans* alters the composition of the matrix. Importantly, matrix from *C. albicans* single-species biofilms is sufficient to induce *S. aureus* biofilm formation and resistance to vancomycin (70).

The antifungal susceptibility of *C. albicans* can also be altered by the presence of bacterial species. In *C. albicans*-*Staphylococcus epidermidis* biofilms, *C. albicans* is more

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resistant to fluconazole and amphotericin B. Interestingly, this enhanced resistance is dependent upon the ability of *S. epidermidis* to produce an extracellular glycocalyx, as the decreased antibiotic sensitivity is not observed in mixed biofilms of the nonglycocalyx variant and *C. albicans* (3, 7). The cooperative interactions between *C. albicans* and glycocalyx-producing *S. epidermidis* in the colonization of abiotic surfaces likely contribute to host infection. Several antifungals diffuse much more rapidly through *C. albicans* biofilms than *S. epidermidis*-*C. albicans* biofilms (8), suggesting that the association of the bacteria in the mixed biofilm may contribute to the increased resistance (3). These studies highlight the fact that interactions between members of a polymicrobial community, like within a mixed-species biofilm on an indwelling device, can have real implications for subsequent infection and efficacy of treatment.

Above we describe interspecies relationships in polymicrobial communities that benefit the involved microorganisms; however, antagonistic *Candida*-bacterium interactions have also been described. El-Azizi and colleagues (55) examined the ability of a number of bacterial species to form biofilms on *C. albicans* and for *C. albicans* to form biofilms on the bacteria. *C. albicans* biofilm development on preformed biofilms of *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter cloacae*, *Pseudomonas aeruginosa* strain PAO1, and *S. epidermidis* (glycocalyx producer) was significantly reduced compared to monospecies *Candida* biofilms. Similar results were obtained when *C. albicans* and the bacteria were added simultaneously. Interestingly, only *K. pneumoniae* and *E. cloacae* experience reduced biofilm formation on preformed *C. albicans* biofilms, suggesting that *C. albicans* was more profoundly affected by the presence of bacteria. It will be important to tease apart the factors that contribute to the inhibition of biofilm formation in these polymicrobial communities to identify targets for development of novel protocols or drug therapies.

POLYMICROBIAL DISSEMINATED INFECTIONS INVOLVING BOTH BACTERIA AND *CANDIDA* SPP.

Polymicrobial systemic infections are common, but their effects on the host, relative to single-species infections, are not well understood. A review of the literature from 1965 to 2004 by Klotz and colleagues (97) revealed that 24% of reported candidemias are accompanied by bacteremia. *C. albicans*, the most commonly reported cause of fungemia, is most frequently accompanied by staphylococci and enterococci, common causative agents of bloodstream infections (BSIs) themselves (97, 167). In a study of 321 candidemic patients from two different hospitals, the frequencies of concurrent bacterial and fungal BSIs were reported to be between 2 and 5% (97). There is a possibility that these numbers are underrepresentative of the true incidence of polymicrobial BSIs due to potential difficulties of obtaining and culturing *Candida* isolates from blood samples that also contain bacteria. Recent PCR-based methods for sensitive detection of *Candida* from biological samples (4) will enable future studies to more accurately calculate the true incidence of coinfection and its correlated patient outcome. A retrospective analysis of 143 patients with simultaneous *Candida* fungemia and bacteremia identified *S. epidermidis* as the predominant bacterial species detected in the blood of *Candida*-infected individuals (10). This observation, combined with the findings that these two organisms readily form robust dual-species biofilms in a static catheter disk biofilm assay (3) and that catheter infections can serve as an important source for BSIs, highlights the need to diagnose and understand polymicrobial biofilm infections.

Clinical evidence suggests that bacterial infection predisposes certain patient groups to fungal BSIs (134) and underscores the importance of in vitro and animal model investigations into the molecular mechanisms that drive these polymicrobial interactions (52). While the clinical significance of simultaneous *Candida*-bacterial disseminated disease is not well understood due to the paucity of retrospective studies and the difficulty of comparing patients with various risk factors, such as underlying disease, there is compelling evidence from animal model work that concurrent *Candida*-bacterial infection results in a poor patient outcome. Murine model studies provide supporting evidence for synergistic effects associated with disseminated infections that contain *C. albicans* and *Staphylococcus aureus*. When mice were challenged with either monomicrobial inocula or a combination of the two species together, it was found that mice infected with either *S. aureus* or *C. albicans* did not succumb to infection at the doses chosen, while the combination was lethal (29, 31). When the sizes of the inocula were alternately adjusted to establish if the presence of either the fungus or the bacterium was responsible for the elevated virulence, it became clear that the contributions were likely not mutual. Reducing the initial *S. aureus* inoculum did not change the ability of sublethal doses of *C. albicans* to infiltrate organs, though the bacterial organ load was significantly reduced. When the *C. albicans* inoculum was lowered to below 10^5 CFU, organ infiltration by either *C. albicans* or *S. aureus* was significantly diminished, leading the investigators to suggest that the presence of *C. albicans* intensifies the virulence of *S. aureus* (30) in this model. Studies where the two species are inoculated sequentially will be of value in determining if one aids in colonization by the other or if the effects are modulated by the host. A retrospective study by Eubanks et al. (58) found that bacteremia often precedes *Candida* BSIs, which is consistent with observations reported by Verghese et al. (161), suggesting that bacterial infection alone, regardless of species, may predispose patients to disseminated candidiasis.

Other opportunistic bacterial pathogens, including *Enterococcus* (*Streptococcus*) *faecalis* and *Serratia marcescens*, in combination with *C. albicans* in a disseminated murine model are correlated with synergistic virulence effects on the host (30, 149). A study by Carlson and Johnson (32) compared the effects of live and heat-killed microbes. Both live and heat-inactivated *C. albicans* organisms support increased *S. aureus* colonization of animals challenged with dual infection. These studies suggest that the effects of *C. albicans* may be through alterations of the host's immune response. Recent studies support the idea that simultaneous exposure to bacterial and fungal infections can influence adaptive immune responses (9), and macrophage function is impaired upon bacterial-*Candida* mixed infection in a rat pneumonia model (149). Carlson and Johnson also found that *S. aureus* is detected mainly at the site of *C. albicans* inoculation, whether intraperitoneal or subcutaneous, but the reciprocal effect is not observed (32). One hypothesis to explain the synergistic effects of mixed *Candida*-bacterium infection is that the presence of *C. albicans* prompts modulation of the host immune response that would ordinarily defend against the invading bacterium, instead providing a protective environment (Fig. 1A). Of course, the converse may occur, as well, as the bacterium may skew the host immune response in favor of *Candida*. A second, but not in-

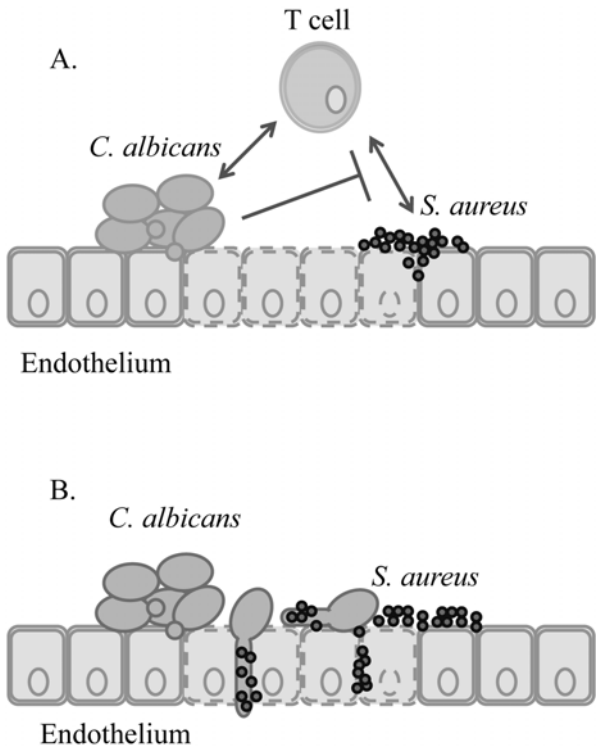

FIGURE 1 Relationships between C. *albicans* and S. *aureus* during infection in a murine model. (A) The presence of C. *albicans* may modulate the host immune response, leading to decreased detection of S. *aureus*. (B) Alternatively, C. *albicans* hyphae may promote increased S. *aureus* colonization and dissemination. Dashed lines represent damage to the endothelial cells. 10.1128/9781555817176.ch20f1

compatible, hypothesis is that as C. *albicans* penetrates and invades host tissues, sublethal doses of the bacteria may gain entry to the bloodstream by directly traversing the damaged tissues or adhering to *Candida* filaments as they protrude through the tissues (Fig. 1B). In vitro tissue models (51, 125) and additional animal studies will be valuable for further dissecting these types of interactions.

ANALYSIS OF C. ALBICANS AND BACTERIA FOUND IN THE ORAL ENVIRONMENT

There are more than 300 microbial species that can be detected on literally every surface in the oral cavity of a healthy individual (129). Imbalance in the normal oral microflora due to antibiotic treatment, poor oral hygiene, or underlying disease often triggers overgrowth of opportunistic pathogens. C. *albicans* is a common inhabitant of the oral mucosa in healthy individuals but can also cause infections in teeth, on the oral mucosa, and on implanted devices, such as dentures and voice prostheses. Colonization is facilitated by the expression of cell surface molecules that promote adhesions to biotic and abiotic surfaces (98, 137, 155) and likely influence the ability to physically interact with other microbes in the oral environment. Studies indicate that the presence of C. *albicans* in infected teeth and plaque is correlated with more severe dental caries in children due to the acid produced by bacterial pathogens (46). Consistent with accumulating clinical data, in vitro studies have revealed that the development of *Candida*-bacterium communities within the oral environment can be central to disease (12).

A number of streptococci are commonly associated with microenvironments in the oral cavity, and mounting evidence suggests that physical interactions between some of these streptococci and C. *albicans* may play an important role in C. *albicans* colonization and disease progression. The ability of C. *albicans* germ tubes to coaggregate with *Streptooccus sanguinis*, S. *gordonii*, S. *oralis*, or S. *anginosus* (90) but not S. *mutans* (80) may facilitate the maintenance of C. *albicans* on dental surfaces, as S. *gordonii* cells form single and multicell cross bridges between C. *albicans* cells (90). Similarly, C. *albicans* coaggregates with S. *gordonii* cells that are preadhered to microtiter dishes, and this aggregation occurs through the concerted efforts of several S. *gordonii* cell surface molecules that mediate interspecies aggregations between S. *gordonii* and other oral microbes (90, 117).

S. *gordonii* cell-associated proteins, SspA and SspB, which bind host integrins and thereby promote phagocytosis by human epithelial cells (122), are important for C. *albicans* coaggregation (81). S. *gordonii* mutants lacking either protein are attenuated in their ability to coaggregate with C. *albicans* (81). In addition, S. *gordonii* cell-linked, fibronectin-binding proteins, CshA and CshB (117), also participate in S. *gordonii*-C. *albicans* coaggregation (81, 127). Salivary proteins that are adsorbed by *Streptococcus* spp. further enhance C. *albicans* binding to S. *gordonii* (128). C. *albicans* expresses cell surface integrin-like molecules (65) and fibronectin adhesins, which mediate binding to the extracellular matrix of the host (142) and may participate in interactions with S. *gordonii*.

Beyond coaggregation, other more complex microbe-microbe interactions likely influence the colonization of oral surfaces. For instance, *Porphyromonas gingivalis*, a gram-negative anaerobe associated with chronic periodontal disease (103), increases adherence of *Streptococcus mutans* to dental surfaces (93). The release of *P. gingivalis* outer membrane vesicles (OMVs) promotes the coaggregation of S. *aureus* and the hyphal form of C. *albicans*, in an in vitro assay, and the presence of *P. gingivalis* OMVs is required for the interaction (94). With fluid flow in the oral cavity, it is likely that *P. gingivalis* OMVs can translocate from the subgingival microenvironments to surfaces where C. *albicans* is present, increasing the ability of S. *aureus* to colonize oral cavities. The presence of *P. gingivalis* and C. *albicans* in the oral cavity may promote high levels of S. *aureus* in the mouth, thereby predisposing at-risk individuals to more serious S. *aureus* infections, such as pneumonia.

INTERACTIONS BETWEEN *C. ALBICANS* AND *PSEUDOMONAS AERUGINOSA, ACINETOBACTER BAUMANII,* AND *BURKHOLDERIA CEPACIA* COMPLEX

Mixed infections containing both *P. aeruginosa* and C. *albicans* are frequently observed in association with contaminated plastic medical devices, in chronic lung infections, and at many other sites in the body (18, 47, 73, 84). Clinical observations suggest that *P. aeruginosa* and C. *albicans* can influence the incidence or relative levels of one another (27, 75, 124). In a study by Azoulay et al. (11), *Candida* colonization was positively correlated with the development of *P. aeruginosa* ventilator-associated pneumonia (VAP) in patients receiving mechanical ventilation, suggesting that the presence of *Candida* may promote the acquisition or

growth of *P. aeruginosa*, which can then cause respiratory disease. In an early study by Adair et al. (2), *C. albicans* and *P. aeruginosa* were more commonly detected together in the endotracheal tubes of VAP patients than in intubated patients without VAP, again suggesting a correlation between cocolonization by both species and VAP. A careful prospective study of patients with cystic fibrosis (CF) found a correlation between mixed infection with both species and lower patient body mass index and lung function, relative to those associated with single-species infections (37). More data are required to determine if these correlations between the presence of both *P. aeruginosa* and *C. albicans* and the onset of disease or worse patient status are due to the fact that *P. aeruginosa-Candida* interactions have negative consequences for the host or if there are host factors that independently affect both the health and prevalence of both species. Direct consequences of mixed infections could include *C. albicans* enhancement of *P. aeruginosa* biofilm formation, antibiotic resistance or virulence factor production, or changes in immune responses or host tissues that result from simultaneous infection by bacteria and fungi.

In vitro interactions may provide insight into the apparent antagonism between *P. aeruginosa* and *C. albicans* in chronic infections that is suggested by increased fungal incidence or fungal loads upon treatment of *P. aeruginosa*-infected patients with antibacterial compounds (27, 95). *P. aeruginosa* can adhere to and form biofilms on *C. albicans* filaments but not on yeast cells, and biofilm formation leads to death of the cell through the action of a secreted phospholipase C that acts on the choline-containing lipids, abundant in fungal membranes (77). Secreted phenazines that can generate reactive oxygen species (ROS) also contribute to fungal killing (66, 78, 95). Although active bacterial biofilm formation on the surface of *C. albicans* cells is required for fungal killing in liquid cocultures (77), *P. aeruginosa* can kill *C. albicans* in the absence of factors needed for biofilm formation when the two species are grown in spatially restricted environments such as on agar plates (66). Both yeast and hyphal forms are killed by phenazines when the two species are forced into close proximity (66), suggesting that contact between bacterium and fungus is more important than biofilm-specific regulation. Gibson et al. (66) also show that *P. aeruginosa* produces a different spectrum of phenazine compounds in the presence of *C. albicans*. 5-Methylphenazinium phenazine-1-carboxylate (5MPCA), which is normally not detected in single-species cultures, is secreted by *P. aeruginosa* in coculture and then taken up and modified within the fungal cytoplasm, causing it to be retained within the cell (66). 5MPCA is more toxic than closely related phenazines, such as pyocyanin, which are not modified within the fungus (66, 118a).

Acinetobacter baumanii, another commonly encountered gram-negative opportunistic pathogen (132), also commonly coinfects with *C. albicans* at a variety of host sites and often in association with catheters and ventilator tubing (35, 144, 147). Similar to the in vitro relationship between *P. aeruginosa* and *C. albicans*, an antagonistic interaction between *C. albicans* and *A. baumannii* has been described (132). In a *Caenorhabditis elegans* polymicrobial infection assay, *A. baumannii* attenuates the virulence of *C. albicans* by repressing its ability to colonize and invade the host (132). *A. baumanii* can attach to the surface of *C. albicans*, and Gaddy et al. identified an *A. baumannii* protein, outer membrane protein A (OmpA), that is essential for attachment to *C. albicans* filaments (64). *Burkholderia cenocepacia* and *C. albicans* (21) also form mixed-species biofilms in liquid medium. In addition, interactions between *C. albicans* and enteric bacteria such as *Escherichia coli*, *S. marcescens* (5, 16, 26, 55, 96), and *Salmonella enterica* serovar Typhimurium have been reported (157). In a *C. elegans* polymicrobial infection model (1, 24, 102), serovar Typhimurium antagonized *C. albicans* by inhibiting its growth and filament and biofilm formation. The interaction seemed to be mediated by a heat-stable, secreted molecule that accumulates in culture supernatants, but the identity of the molecule is not yet known.

Gram-negative bacteria also affect *C. albicans* morphology. *C. albicans* cells secrete a small molecule, farnesol, which represses hyphal growth even in the presence of signals that normally trigger hyphal growth (82). The *C. albicans* pathways that are affected by farnesol are described in detail below. Interestingly, a cell-cell signaling molecule produced by *P. aeruginosa*, 3-oxo-C12-homoserine lactone (3OC12HSL), has a similar effect on *C. albicans* (78, 116). *P. aeruginosa* 3OC12HSL may be particularly effective as it accumulates in biofilms (33) such as those that form on *C. albicans* hyphae (78). The *C. albicans* response to 3OC12HSL may be due to perturbations of the *C. albicans* Ras1 signaling pathway (45) based on the activity of structurally related molecules, such as dodecanol and *C. albicans*-produced farnesol, and the fact that filamentation in response to activators of Ras1 signaling is perturbed by 3OC12HSL. Other gram-negative bacteria, such as *B. cenocepacia* (21) and *Xanthomonas campestris* (163), produce compounds that similarly repress *C. albicans* hyphal growth. *P. aeruginosa*, *B. cenocepacia*, and *X. campestris* are all commonly found in soils, where they likely interact with or compete with fungi. It is possible that the molecules that affect *C. albicans* morphology are also involved in other bacterial-fungal interactions. Below, we discuss how farnesol has a range of effects on bacteria, including *P. aeruginosa*.

MOLECULAR MECHANISMS OF INTERACTIONS BETWEEN *CANDIDA* AND GRAM-POSITIVE BACTERIA

While *C. albicans* frequently encounters large numbers of gram-positive bacteria in the oral, intestinal, and skin microfloras, a relatively small number of studies describe the molecular interactions that occur between *C. albicans* and gram-positive microbes. Recent work by Bamford and colleagues (15) has taken a first step in identifying and characterizing the cell signaling events involved in *C. albicans-S. gordonii* biofilm formation on abiotic surfaces. Not only does *S. gordonii* stimulate the development of *C. albicans* biofilms when both are added simultaneously, but also preformed *S. gordonii* biofilms provide a surface for more robust *Candida* biofilm formation, and addition of the bacteria to preformed *C. albicans* biofilms enhances size. These effects are partially mediated by *S. gordonii* surface proteins SspA and SspB (15). *S. gordonii* supernatants also promote biofilm formation and hyphal growth, while *S. gordonii luxS* mutants that are unable to produce the bacterial signaling molecule autoinducer 2 (AI-2) do not. Simultaneous addition of an *S. gordonii luxS* mutant, *C. albicans*, and a synthetic AI-2 precursor, 4,5-dihydroxy 2,3-pentanedione (DPD), does not rescue biofilm formation, suggesting that either DPD cannot substitute for AI-2 or another factor produced by wild-type *S. gordonii* contributes to *C. albicans* biofilm growth (15). *C. albicans* hyphal growth is induced in response to a number of environmental signals via several pathways, including the Ras1-cyclic AMP (Ras1-cAMP) and Cek1 mitogen-activated protein (MAP) kinase cascades (for a review, see

reference 20). Coincubation of *S. gordonii* and *C. albicans* results in phosphorylation of Cek1, suggesting that the physical and/or chemical interactions between the microbes promote *C. albicans* hyphal growth via activation of the Cek1 pathway, though the stimulus and signal receptor are not known; H_2O_2 alone, an *S. gordonii* metabolite, was not sufficient to induce Cek1 phosphorylation (15).

Often it is not only physical microbe-microbe interactions that influence community dynamics but also factors produced by microbes in the community that accumulate and influence intraspecies and interspecies relationships and behaviors. The Ras1-cAMP signaling pathway is required for the induction of *C. albicans* hyphal growth by serum (62), and surprisingly, the components in blood serum that are primarily responsible for signal induction are byproducts from the degradation of bacterial cell wall peptidoglycan (PGN) (168). A component of the cell walls of both gram-positive and gram-negative bacteria, PGN is broken down during normal cell replication, releasing muramyl dipeptides (MDPs) into the surrounding environment. Both *E. coli*- and *S. aureus*-derived MDPs and chemically synthesized MDPs stimulate robust *C. albicans* hypha induction. Comprehensive biochemical analyses further demonstrate that MDPs directly bind to a leucine-rich repeat region of the *C. albicans* adenylate cyclase (Cyr1), promoting cAMP synthesis and induction of hyphal growth (168). Mutation of components of the Ras1-cAMP pathway renders cells attenuated for virulence in murine models (109, 118, 150); therefore, identifying novel targets that regulate this key process is of the utmost importance as we strive to develop better tools to control *Candida*-associated diseases. While it still remains a bit of a mystery as to how bacterially derived molecules find their way into the bloodstream, these studies bring up the question of whether treatment with antibiotics that cause cell lysis and release of PGN fragments could contribute to increased *C. albicans* virulence.

INTERSPECIES INTERACTIONS AND NAC-BACTERIUM INTERACTIONS

While non-*C. albicans Candida* (NAC) species have emerged as important causes of invasive disease (136), there is a paucity of studies investigating microbe-microbe interactions involving NACs. A small study of the oral microflora in individuals with denture stomatitis found that 75% were positive for *C. albicans*, and a full quarter of these were also positive for *Candida glabrata* (38). *C. glabrata* was detected never singly but always in combination with *C. albicans*. In vitro, *C. glabrata* forms more robust biofilms in coculture with *C. albicans* than when it is grown in monoculture, suggesting that interaction with *C. albicans* has a beneficial effect on *C. glabrata* colonization and persistence in oral communities (71, 133, 164).

Like *C. albicans*, *C. glabrata* is also a frequent cause of BSIs (134, 136, 138, 167), and these BSIs often occur in combination with pathogenic bacteria, including *Escherichia coli* (134). While dual infection with *C. albicans* and *E. coli* or *E. coli* lipopolysaccharide (LPS) is more lethal than *C. albicans* alone (5, 26), mice with longstanding *C. glabrata* systemic infections that are subsequently challenged with *E. coli* or *E. coli*-derived LPS experience decreased fungal burden in the kidneys and liver in a reproducible, but nonstatistically significant, manner compared to mice challenged with *C. glabrata* alone (74). Larger studies may be needed to determine the importance of this effect, and the differences, if any, between the effects of *E. coli* LPS on animals infected with *C. albicans* or *C. glabrata*. One potential explanation for differences lies in the host response to *E. coli* LPS. Tumor necrosis factor alpha production in response to LPS leads to activation of macrophages and increased phagocytosis of *C. glabrata* and possibly *C. albicans* (74). When *C. albicans* yeast cells are phagocytosed by macrophages, they rapidly undergo morphogenesis and escape (41, 110); however, the inability of *C. glabrata* to readily form filaments may prevent its escape, resulting in decreased fungal burden. *E. coli* LPS does not seem to physically affect *C. glabrata* colonization. While Bandara et al. (16) found that neither *C. glabrata* nor *C. albicans* biofilm formation is altered by the presence of *E. coli* or LPS, *E. coli* biofilm formation is significantly enhanced by the presence of *C. glabrata* (16). When live *E. coli* is used, however, biofilm inhibition is observed (158), which highlights the complex nature of these multifactorial interactions. With growing evidence that antibiotic treatment is frequently associated with increased fungal load (79) and may liberate significant LPS from resident gram-negative bacteria (59), future studies investigating the effects of LPS from a range of gram-negative bacteria on the physiology of NACs will be valuable.

Candida dubliniensis is often associated with oropharyngeal candidiasis in individuals with human immunodeficiency virus. While *C. dubliniensis* is not a normal constituent of the oral cavity of healthy individuals, interactions with pathogenic bacteria in the mouth may facilitate oral colonization. *C. dubliniensis* clinical isolates, but not *C. albicans*, are able to coaggregate with the gram-negative bacterium *Fusobacterium nucleatum* in vitro (86). *F. nucleatum* plays a pivotal role in microbial colonization of the oral cavity, due to its inherent capacity for binding to host epithelial cells (68), and thus has the potential to act as a structural intermicrobial bridge for less adherent *Candida* spp. With indications that repeated antifungal treatment is a predisposing cause of colonization by resistant NACs due to emergent antifungal resistance (119), interactions such as those highlighted above may have significant clinical implications for certain patient groups (86, 156).

THE ROLE OF PROBIOTICS IN THE CONTROL OF CANDIDIASIS

Interest in the use of probiotics to combat disease-causing microbes has expanded over the past decade. Prosthetic voice implants made of silicone rubber are commonly colonized by *C. albicans* biofilms, and these biofilms not only act as a source for infection of surrounding tissues but also destroy the silicone material and restrict airflow through the airways (56, 57). *Streptococcus thermophilus* B produces biosurfactants composed of a mixture of lipids and glycolipids that inhibit the initial adherence of *C. albicans* and *Candida tropicalis* (28). Furthermore, both *Lactococcus lactis* 53 and *S. thermophilus* A, two probiotic bacteria normally found in dairy products, also inhibit biofilm formation by *C. albicans* and *C. tropicalis* on voice implants in an artificial throat model (99, 107). Whether these probiotic species can be used successfully to protect these prosthetic devices is unclear; nevertheless, these and related studies may identify new compounds that can be applied to abiotic surfaces to inhibit *Candida* colonization or invasion.

A series of clinical trials have investigated the use of probiotics for the management of recurrent vulvovaginal candidiasis (for a review, see reference 60), and while the data

are inconclusive, further in vitro and animal studies are warranted. *C. albicans* is responsible for over 85% of reported cases of vulvovaginal candidiasis, with *C. glabrata* the second most common causative agent (130). As *C. glabrata* is inherently resistant to azoles, the discovery that endogenous vaginal lactobacilli produce metabolites with activity against *C. glabrata* and *C. albicans* (92, 146) provides an untapped resource for treating infection. Overgrowth of pathogenic microbes in the vagina is partially controlled by the presence of lactic acid bacteria that contribute to the maintenance of a low environmental pH. In fact, when naturally occurring lactobacilli collected from vaginas of healthy women were fed to mice with an oral *C. albicans* infection, the fungus was cleared and no residual *Lactobacillus acidophilus* remained in the oral cavity (54). Immunodeficient mice that ordinarily succumb to infection by *C. albicans* are protected when several species of lactobacilli and *Bifidobacterium infantis* are applied to oral or anal surfaces (162). Lactic acid bacteria may affect *Candida* community formation on abiotic surfaces and within the host. Lactobacilli have been implicated in the suppression of disease progression by *C. albicans*. First, vaginal lactobacilli are thought to adhere to host epithelial cells, preventing colonization by the fungus (22, 40). Second, the presence of lactobacilli alters the host immune response and prevents *C. albicans* disease progression (154). Finally, short-chain fatty acids, such as butyric acid, that are produced by lactobacilli are sufficient to inhibit hyphal growth of *C. albicans* (123). When either live bacteria or culture supernatants were cultured with *C. albicans*, serum-induced filamentation was inhibited. Interestingly, culture supernatants were more potent repressors than live bacteria (123).

CANDIDA QUORUM SENSING IN SINGLE-SPECIES POPULATIONS AND INTERSPECIES INTERACTIONS

Unicellular organisms often produce small, diffusible chemical signals, referred to as quorum-sensing molecules (QSMs), that coordinate group behavior within single-species populations. QSMs accumulate in the local environment as the cell density increases; once threshold concentrations are reached, specific QSM-responsive pathways mediate the induction of genes involved in diverse processes such as biofilm formation, group motility, and virulence. The intraspecies signaling often also controls processes that are critical for competition within polymicrobial communities (33, 72), and bacterial QSMs have been documented to mediate interspecies communication (43).

For decades, researchers were aware of potential QSMs in *C. albicans* cultures. A factor(s) in culture supernatants was known to suppress *C. albicans* morphogenesis in response to hypha inducers. In 2001, Hornby et al. (82) identified that key regulatory factor as a 16-carbon sesquiterpene, *E,E*-farnesol. The identification of farnesol marked the first description of a QSM produced by a fungus, and this finding opened the door to further study of mechanisms by which *C. albicans* regulates coordinated cell behaviors at the population level in single-species and mixed-species situations (104). While some *C. dubliniensis* strains produce levels of farnesol that are comparable to those found in *C. albicans* cultures, farnesol is detected only at much lower concentrations in *C. parapsilosis*, *C. tropicalis*, *C. krusei*, *C. glabrata*, and *C. guilliermondii* cultures (164).

Like bacterial QSMs, farnesol is constitutively released (by *C. albicans* cells), and it accumulates in the extracellular milieu as the cell density increases. Supernatant farnesol concentrations typically range from 10 to 60 μM (164), but localized concentrations within biofilms are likely greater, as has been shown for bacterial QSMs (33). While most *C. albicans* strains produce farnesol at comparable concentrations, at least one strain of *C. albicans*, 10231, has been shown to produce an alternative molecule, farnesoic acid (83, 126). Farnesol at a concentration of 30 μM can inhibit the induction of filamentation in 50% of cells in GPP medium (82), but not surprisingly, it has been documented that culture conditions and inoculum preparation affect the potency and effects of farnesol (105, 152, 160). The regulatory outputs of farnesol signals that are relevant to *C. albicans* behavior and survival in microbial communities are discussed further below. In addition to farnesol, *C. albicans* produces other metabolites that accumulate in the culture medium and may also serve as QSMs. Tyrosol, an amino acid alcohol, accelerates the induction of hyphal development under filament-inducing conditions and reduces lag phase when yeast cells from stationary-phase cultures are diluted to low density in fresh media (6, 34). Furthermore, the alcohols 1-dodecanol, isoamyl alcohol, 2-phenylethanol, and nerolidol accumulate in culture supernatants and inhibit morphogenesis at physiologically relevant concentrations (115). While all of these known and potential QSMs may play important roles in *C. albicans* fitness within the host, only farnesol has been tested for its importance in in vivo infections (121). The remainder of this chapter focuses on farnesol signaling in *C. albicans* populations and *Candida*-bacterial cross talk involving farnesol.

Autoregulation in *C. albicans* Single-Species Populations by Farnesol

While a specific receptor for farnesol has yet to be detected, studies indicate that it is likely that farnesol affects *C. albicans* in multiple ways. There are robust data suggesting that an important target of farnesol is the small GTPase Ras1 (45, 49). The Ras family of GTPases is highly conserved throughout eukaryotes and regulates many key processes (53) acting as a molecular switch, turning signals on and off. In *C. albicans*, activated Ras1 interacts with adenylate cyclase (Cyr1) (61), leading to cAMP activation of protein kinase A (PKA) and morphogenesis. In response to stimuli that activate the Ras1-cAMP-PKA signaling cascade (36, 113) but not the MAP kinase cascade (45), farnesol inhibits *C. albicans* morphogenesis. Addition of a nonhydrolyzable cAMP analog, dibutyryl cAMP, rescues filamentation in farnesol-treated cells, and a constitutively filamentous strain bearing a hyperactivated $Ras1^{G13V}$ protein is sensitive to farnesol. These effects are independent of phosphodiesterase activity mediated by Pde1 and Pde2 (45). Taken together, these data demonstrate that a central target of farnesol is the Ras1-cAMP signaling pathway. There are several mechanisms by which farnesol may directly affect Ras1 signaling. Ras1 becomes membrane associated due to a farnesyl modification, and farnesol may compete with farnesyl pyrophosphate, preventing this modification and membrane localization. Farnesol is a lipophilic molecule, and disruption of the membrane environment may prevent the proper localization of Ras1 and interactions with activators or effectors. A third, but not incompatible, possibility is that farnesol interferes with protein-protein interactions, precluding the association of Ras1 with activators, effectors, or signaling partners. Further biochemical and genetic analyses with strains bearing Ras1 proteins that are differentially prenylated will be useful in identifying the molecu-

lar mechanism of farnesol action on Ras1 signaling in C. *albicans*.

Several other proteins have been linked to farnesol sensing in C. *albicans*. The histidine kinase Chk1 is part of a two component signal transduction system in C. *albicans* that regulates cell wall biogenesis and response to oxidative stress (108). Concentrations of farnesol that inhibit filamentation and biofilm formation of the parental wild-type control do not significantly repress these phenotypes in the *chk1/chk1* mutant (100). Interestingly, culture supernatants from overnight cultures of the *chk1/chk1* mutant do not inhibit biofilm formation of the parental wild-type strain, while wild-type culture supernatants applied to wild-type cells cause a threefold reduction in biofilm development (145). These data suggest that *chk1/chk1* does not accumulate farnesol in overnight culture supernatants; however, analysis of farnesol levels has not been reported. The expression of C*HK1* is reported to be negatively regulated by the Tup1 transcriptional repressor, which is also an important negative regulator of many genes involved in morphogenesis (23, 91). The *tup1/tup1* mutant is constitutively filamentous and is insensitive to farnesol-mediated inhibition of filamentation (100). High levels of oxidative stress trigger the phosphorylation of the Hog1 MAP kinase, leading to upregulation of genes involved in the stress response. Farnesol treatment of wild-type cells also promotes sustained phosphorylation of Hog1 in the absence of additional oxidative stressors (153). Finally, the Cek1 MAP kinase is phosphorylated upon resumption of growth when cells from overnight stationary-phase cultures are resuspended in fresh media, and 200 μM farnesol inhibits phosphorylation, independent of Chk1(145). Cross talk between the Ras1-cAMP pathway, the Chk1 two-component signal transduction pathway, the Cek1 MAP kinase pathway, and the Hog1 MAP kinase cascade has been reported (49, 106); however, it is still unclear if farnesol acts on each pathway independently or if Ras1 is the principal farnesol sensor.

A range of important phenotypes are modulated by physiological concentrations of farnesol, including filamentation and biofilm formation (82, 140), generation of ROS (49, 152), and resistance to oxidative stress and heat stress (45, 49, 105, 165). The inherent ability to interconvert between the yeast and hyphal growth forms plays a role in colonization, virulence, and survival in vivo (14, 17, 106, 150, 169). Thus, the regulation of morphology by farnesol, likely through effects on the Ras1-cAMP signaling pathway, may affect many phenotypes relevant to disease. Morphogenesis is also crucial for biofilm formation (141), which proceeds through a series of regulated stages beginning with adherence to a surface, followed by the development of a complex population of yeast cells, hyphae, and pseudohyphae. The accumulation of farnesol within a biofilm may promote the release of yeast cells that can seed new environments. Biofilm-released cells are primed for colonizing new surfaces, as they are better able to form biofilms, colonize epithelial cells, and cause disease in mice (159). The effect of farnesol on Ras1-cAMP signaling also has the potential to affect antifungal resistance (88). To make matters more complex, the presence of subinhibitory concentrations of the commonly used azole drug fluconazole, which targets the sterol biosynthetic pathway, leads to increased production of farnesol (83). This regulation of quorum sensing may be a way that *Candida* modulates its behavior in response to stresses encountered in the host and within microflora communities.

Farnesol repression of Ras1 signaling also affects C. *albicans* population-level responses to oxidative stress. The first line of host defense against an invasive C*andida* infection is mediated by macrophages and neutrophils that utilize an arsenal of noxious molecules to battle the invading fungus, not the least of which are ROS (143). Detoxification of hydrogen peroxide by catalases is a common mechanism for cellular resistance to ROS, and inhibition of the Ras1-cAMP signaling pathway in C. *albicans*, either due to mutation of genes encoding Ras1 or adenylate cyclase or via the addition of farnesol, reduces sensitivity to oxidative stress by derepressing C*AT1*, leading to high levels of catalase (13, 45, 49, 69, 166). While previous studies have demonstrated that the histidine kinase Chk1 also plays a role in the response to farnesol (100) and the MAP kinase Hog1 shows increased phosphorylation in response to farnesol (153), neither was required for farnesol-mediated protection of H_2O_2 stress (49).

As mentioned above, the effects of farnesol can vary with conditions. While farnesol levels that inhibit 100% filamentation in wild-type cells have been reported to have no effects on growth rate or viability (45, 82, 105), some studies find growth inhibition and cell death in the presence of farnesol (152, 160). Interestingly, in addition to a protective effect against ROS, farnesol can trigger the accumulation of ROS within C. *albicans* cells (152), particularly when added to cells from exponential-phase cultures (49). Thus, it is possible that farnesol-mediated downregulation of the Ras1-cAMP signaling pathway, leading to increased catalase levels, protects cells from ROS that are concurrently generated by farnesol, perhaps through effects on the mitochondria (111). Alternatively, the generation of ROS by farnesol itself may be beneficial. Pretreatment with subinhibitory concentrations of H_2O_2 may prime C. *albicans* cells for enhanced resistance to subsequent H_2O_2 exposure (89). The increased resistance to oxidative stress induced by farnesol may reflect one of the ways C. *albicans* quorum sensing increases fitness, as ROS will be encountered during a host immune response and within polymicrobial communities containing H_2O_2-producing bacteria like lactobacilli or streptococci (63).

Farnesol Effects on Other Fungi

C. *albicans*- and C. *albicans*-produced farnesol inhibits aspergilli, and this antagonism may provide important insight into ways to treat or control different A*spergillus* infections that commonly arise in hospital settings. Among the human fungal pathogens, A*spergillus fumigatus* is a leading cause of invasive mycoses (136), and it is responsible for pulmonary destruction and disseminated disease predominantly in the severely immunocompromised. While farnesol inhibits A. *fumigatus* growth and filamentation (50, 151), it does so at concentrations above biologically relevant levels. In contrast, A. *nidulans* displays a higher sensitivity to farnesol than A. *fumigatus*. Semighini and colleagues (151) report that at 10 μM farnesol, A. *nidulans* exhibits apoptotic markers, including phosphatidylserine exposure and DNA condensation and fragmentation. The induction of this apoptotic-like response requires functional mitochondria, as blocking mitochondrial function with oligomycin completely protects against farnesol-induced apoptosis. Recent clues to the target of farnesol in A. *fumigatus* point to the G-protein complex containing the Gα subunit, FadA, as mutants with a hyperactivated G-protein signaling cascade are hypersensitive to farnesol, while those lacking the positive regulator of the complex are resistant (50). The data of Dichtl and colleagues (50) suggest that the farnesol-induced cell death is not due solely to ROS generation, as mutants

lacking kinases important for the cell wall integrity pathway are hypersensitive to farnesol yet do not accumulate ROS as wild-type cells do.

Similar observations have been made with other fungal pathogens, including the etiologic agent of *Pneumocystis* pneumonia in the lungs of immunocompromised individuals or those with underlying health issues, such as chronic obstructive pulmonary disease (COPD) (120). Farnesol caused a 98% reduction in metabolic activity in *Pneumocystis* biofilms (44) and inhibited morphogenesis in *Paracoccidiodes brasiliensis* (48). Further examination of *P. brasiliensis* internal cell structures demonstrated that while the cell wall was intact, significant degradation of internal organelles resulted (48). The mechanisms for farnesol effects on *Pneumocystis* and *P. brasiliensis* remain to be determined. Farnesol also causes a temporary growth arrest in *C. parapsilosis* (148) through unknown pathways. Many of the effects of farnesol on other fungi may be explained by action of farnesol on the mitochondria. Farnesol induces mitochondrion-dependent generation of ROS in *Saccharomyces cerevisiae* potentially by disrupting electron transfer (112) or by altering the mitochondrial proton gradient and triggering transport of protons from the matrix to the intermembrane space, resulting in polarization of the mitochondrial membrane and ROS production (111).

Farnesol in *Candida*-Bacterium Communities

As described above, there is a report that CF patients show a steeper decline in lung function after the detection of *C. albicans* in their sputum, though a direct causal relationship has not been established (37). While *C. albicans* rarely causes invasive lung disease, these studies bring up the possibility that the presence of *C. albicans* in the lung may exacerbate an already grave *P. aeruginosa* infection. In laboratory studies, coculture with *C. albicans* leads to increased *P. aeruginosa* virulence factor production (66). In addition, *P. aeruginosa* sputum isolates from individuals with CF often acquire adaptive mutations that lead to a loss of LasR function (76), and culture of these isolates either with *C. albicans* colony biofilms or on solid media containing farnesol results in a significant upregulation of the virulence factors controlled by *P. aeruginosa* QSMs (Fig. 2, left). In LasR$^-$ cells, farnesol leads to increased levels of two *P. aeruginosa* QSMs, C4-homoserine lactone and *Pseudomonas* quinolone signal (PQS), which translates into high levels of phenazine and coregulated virulence factors (42a). The effects of farnesol on virulence factor regulation are dependent on the culture conditions. In contrast to what is observed in colony biofilms, farnesol inhibits the production of the PQS in cells from early-log-phase cultures (Fig. 2, right). Farnesol likely interferes with a productive transcription factor-promoter association between PqsR and the *pqsABCDE* promoter (42), and this early inhibition of PQS production may in fact promote *P. aeruginosa* biofilm formation (116). In addition to farnesol effects on *P. aeruginosa*, farnesol-containing supernatants from *C. albicans* cultures inhibited *A. baumanii* biofilm growth (131, 132) and decreased *E. coli* resistance to polymyxin B (25).

C. albicans-produced farnesol can influence gram-positive bacteria in a variety of ways. Biofilm formation by both methicillin-sensitive *S. aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) is inhibited by farnesol (87), yet farnesol only alters antibiotic sensitivity of MSSA strains, not MRSA strains, in a disk diffusion assay (87). Farnesol treatment of preformed MSSA biofilms, in combination with subinhibitory antibiotic concentrations, caused biofilm disruption (87). The authors suggest that the synergism observed between farnesol and the antibiotics is due to the ability of farnesol to compromise *S. aureus* membrane permeability, as demonstrated by an ethidium bromide uptake assay (87). Similarly, it has been shown that upon treatment with biologically relevant levels of farnesol (~50 μM), there is a rapid efflux of K^+ ions, indicative of membrane damage (85). Brehm-Stecher and Johnson (25) found that higher concentrations of farnesol (1 mM) inhibited *S. aureus* growth, while Kuroda et al., using higher concentrations of farnesol (>1 mM for MSSA and >10 mM for MRSA), saw increased susceptibility to a number of antibiotics that they tested (101). In addition, Makoto et al. (114) found that farnesol inhibits production of extracellular lipases. Because staphylococci can colonize indwelling devices (139), the fact that farnesol makes *S. aureus* biofilms more sensitive to antibiotics may indicate that treatment with farnesol or related molecules will possibly be a way to render antibiotics more effective.

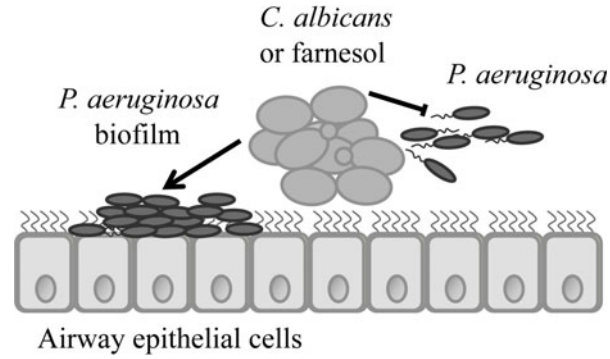

FIGURE 2 *C. albicans* and *C. albicans*-produced farnesol affects *P. aeruginosa* virulence in multiple ways. In biofilms, farnesol promotes upregulation of virulence factor production, particularly in *P. aeruginosa* LasR-defective strains, which are commonly observed in chronic lung infections associated with CF (left). When *P. aeruginosa* wild-type or LasR$^-$ strains are at low densities, *C. albicans*-produced farnesol inhibits virulence factor production but may promote biofilm formation (right).

10.1128/9781555817176.ch20f2

CONCLUSIONS

The estimated number of cases of candidemia in the United States rose by 52% between 2000 and 2005, increasing to between 10,500 and 42,000 cases per year, with an accompanying 40% mortality rate (136). Recognition that bacteria are often found in association with *Candida* in mixed-species biofilms and that *C. albicans* BSIs can be polymicrobial should drive future studies aimed at characterizing these interactions and understanding the best treatment options. Other infections, such as those in the mouth, the lungs, or the female reproductive tract, may be directly affected by physical and chemical interactions between bacteria and fungi, and these interactions potentially reveal ways that *Candida* pathogenesis can be modulated or prevented. The studies on mixed-species infections may also suggest that the presence of a microbe modulates the host immune response such that other opportunistic microbes in the microenvironment are not effectively cleared by macrophages and neutrophils. If further animal model studies find this to be the case, different prophylaxis strategies, perhaps with antifungals or probiotics, may be of value in controlling

hospital infections. The interactions between *Candida* and bacteria and their combined effects on the host are likely multifaceted and will require the development of new model systems to dissect the complex exchanges that drive disease.

REFERENCES

1. **Aballay, A., P. Yorgey, and F. M. Ausubel.** 2000. *Salmonella typhimurium* proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Curr. Biol.* **10:**1539–1542.
2. **Adair, C. G., S. P. Gorman, B. M. Feron, L. M. Byers, D. S. Jones, C. E. Goldsmith, J. E. Moore, J. R. Kerr, M. D. Curran, G. Hogg, C. H. Webb, G. J. McCarthy, and K. R. Milligan.** 1999. Implications of endotracheal tube biofilm for ventilator-associated pneumonia. *Intensive Care Med.* **25:**1072–1076.
3. **Adam, B., G. S. Baillie, and L. J. Douglas.** 2002. Mixed species biofilms of *Candida albicans* and *Staphylococcus epidermidis*. *J. Med. Microbiol.* **51:**344–349.
4. **Ahmad, S., Z. Khan, A. S. Mustafa, and Z. U. Khan.** 2002. Seminested PCR for diagnosis of candidemia: comparison with culture, antigen detection, and biochemical methods for species identification. *J. Clin. Microbiol.* **40:** 2483–2489.
5. **Akagawa, G., S. Abe, and H. Yamaguchi.** 1995. Mortality of *Candida albicans*-infected mice is facilitated by superinfection of *Escherichia coli* or administration of its lipopolysaccharide. *J. Infect. Dis.* **171:**1539–1544.
6. **Alem, M. A. S., M. D. Y. Oteef, T. H. Flowers, and L. J. Douglas.** 2006. Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development. *Eukaryot. Cell* **5:**1770–1779.
7. **Al-Fattani, M. A., and L. J. Douglas.** 2006. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. *J. Med. Microbiol.* **55:**999–1008.
8. **Al-Fattani, M. A., and L. J. Douglas.** 2004. Penetration of *Candida* biofilms by antifungal agents. *Antimicrob. Agents Chemother.* **48:**3291–3297.
9. **Allard, J. B., L. Rinaldi, M. J. Wargo, G. Allen, S. Akira, S. Uematsu, M. E. Poynter, D. A. Hogan, M. Rincon, and L. A. Whittaker.** 2009. Th2 allergic immune response to inhaled fungal antigens is modulated by TLR-4-independent bacterial products. *Eur. J. Immunol.* **39:** 776–788.
10. **Alonso-Valle, H., O. Acha, J. D. García-Palomo, C. Fariñas-Álvarez, C. Fernández-Mazarrasa, and M. C. Fariñas.** 2003. Candidemia in a tertiary care hospital: epidemiology and factors influencing mortality. *Eur. J. Clin. Microbiol. Infect. Dis.* **22:**254–257.
11. **Azoulay, E., J.-F. Timsit, M. Tafflet, A. de Lassence, M. Darmon, J.-R. Zahar, C. Adrie, M. Garrouste-Orgeas, Y. Cohen, B. Mourvillier, B. Schlemmer, and the Outcomerea Study Group.** 2006. *Candida* colonization of the respiratory tract and subsequent *Pseudomonas* ventilator-associated pneumonia. *Chest* 129:110–117.
12. **Baena-Monroy, T., V. Moreno-Maldonado, F. Franco-Martinez, B. Aldape-Barrios, G. Quindos, and L. Sanchez-Vargas.** 2005. *Candida albicans*, *Staphylococcus aureus* and *Streptococcus mutans* colonization in patients wearing dental prosthesis. *Med. Oral Patol. Oral Cir. Bucal* **10:** E27–E39.
13. **Bahn, Y.-S., M. Molenda, J. F. Staab, C. A. Lyman, L. J. Gordon, and P. Sundstrom.** 2007. Genome-wide transcriptional profiling of the cyclic AMP-dependent signaling pathway during morphogenic transitions of *Candida albicans*. *Eukaryot. Cell* **6:**2376–2390.
14. **Bahn, Y. S., and P. Sundstrom.** 2001. Cap1, an adenylate cyclase-associated protein gene, regulates bud-hypha transitions, filamentous growth, and cyclic AMP levels and is required for virulence of *Candida albicans*. *J. Bacteriol.* **183:**3211–3223.
15. **Bamford, C. V., A. d'Mello, A. H. Nobbs, L. C. Dutton, M. M. Vickerman, and H. F. Jenkinson.** 2009. *Streptococcus gordonii* modulates *Candida albicans* biofilm formation through intergeneric communication. *Infect. Immun.* **77:** 3696–3704.
16. **Bandara, H. M. H. N., J. Y. Y. Yau, R. M. Watt, L. J. Jin, and L. P. Samaranayake.** 2009. *Escherichia coli* and its lipopolysaccharide modulate *in vitro Candida* biofilm formation. *J. Med. Microbiol.* **58:**1623–1631.
17. **Banerjee, M., D. S. Thompson, A. Lazzell, P. L. Carlisle, C. Pierce, C. Monteagudo, J. L. Lopez-Ribot, and D. Kadosh.** 2008. Ume6, a novel filament-specific regulator of *Candida albicans* hyphal extension and virulence. *Mol. Biol. Cell* **19:**1354–1365.
18. **Bauernfeind, A., G. Emminger, G. Horl, S. Ott, B. Przyklenk, and C. Weisslein-Pfister.** 1987. Bacteriological effects of anti-*Pseudomonas aeruginosa* chemotherapy in cystic fibrosis. *Infection* **15:**403–406.
19. **Beenken, K. E., J. S. Blevins, and M. S. Smeltzer.** 2003. Mutation of *sarA* in *Staphylococcus aureus* limits biofilm formation. *Infect. Immun.* **71:**4206–4211.
20. **Biswas, S., P. Van Dijck, and A. Datta.** 2007. Environmental sensing and signal transduction pathways regulating morphopathogenic determinants of *Candida albicans*. *Microbiol. Mol. Biol. Rev.* **71:**348–376.
21. **Boon, C., Y. Deng, L. H. Wang, Y. He, J. L. Xu, Y. Fan, S. Q. Pan, and L. H. Zhang.** 2008. A novel DSF-like signal from *Burkholderia cenocepacia* interferes with *Candida albicans* morphological transition. *ISME J.* **2:** 27–36.
22. **Boris, S., J. E. Suarez, F. Vazquez, and C. Barbes.** 1998. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. *Infect. Immun.* **66:**1985–1989.
23. **Braun, B. R., and A. D. Johnson.** 1997. Control of filament formation in *Candida albicans* by the transcriptional repressor *TUP1*. *Science* **277:**105–109.
24. **Breger, J., B. B. Fuchs, G. Aperis, T. I. Moy, F. M. Ausubel, and E. Mylonakis.** 2007. Antifungal chemical compounds identified using a *C. elegans* pathogenicity assay. *PLoS Pathog.* **3:**e18.
25. **Brehm-Stecher, B. F., and E. A. Johnson.** 2003. Sensitization of *Staphylococcus aureus* and *Escherichia coli* to antibiotics by the sesquiterpenoids nerolidol, farnesol, bisabolol, and apritone. *Antimicrob. Agents Chemother.* **47:** 3357–3360.
26. **Burd, R. S., C. S. Raymond, and D. L. Dunn.** 1992. Endotoxin promotes synergistic lethality during concurrent *Escherichia coli* and *Candida albicans* infection. *J. Surg. Res.* **52:**537–542.
27. **Burns, J. L., J. M. Van Dalfsen, R. M. Shawar, K. L. Otto, R. L. Garber, J. M. Quan, A. B. Montgomery, G. M. Albers, B. W. Ramsey, and A. L. Smith.** 1999. Effect of chronic intermittent administration of inhaled tobramycin on respiratory microbial flora in patients with cystic fibrosis. *J. Infect. Dis.* **179:**1190–1196.
28. **Busscher, H. J., C. G. van Hoogmoed, G. I. Geertsema-Doornbusch, M. van der Kuijl-Booij, and H. C. van der Mei.** 1997. *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp. on silicone rubber. *Appl. Environ. Microbiol.* **63:**3810–3817.
29. **Carlson, E.** 1983. Effect of strain of *Staphylococcus aureus* on synergism with *Candida albicans* resulting in mouse mortality and morbidity. *Infect. Immun.* **42:**285–292.

30. **Carlson, E.** 1983. Enhancement by *Candida albicans* of *Staphylococcus aureus*, *Serratia marcescens*, and *Streptococcus faecalis* in the establishment of infection in mice. *Infect. Immun.* **39:**193–197.
31. **Carlson, E.** 1982. Synergistic effect of *Candida albicans* and *Staphylococcus aureus* on mouse mortality. *Infect. Immun.* **38:**921–924.
32. **Carlson, E., and G. Johnson.** 1985. Protection by *Candida albicans* of *Staphylococcus aureus* in the establishment of dual infection in mice. *Infect. Immun.* **50:**655–659.
33. **Charlton, T. S., R. de Nys, A. Netting, N. Kumar, M. Hentzer, M. Givskov, and S. Kjelleberg.** 2000. A novel and sensitive method for the quantification of *n-3*-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. *Environ. Microbiol.* **2:**530–541.
34. **Chen, H., M. Fujita, Q. Feng, J. Clardy, and G. R. Fink.** 2004. Tyrosol is a quorum-sensing molecule in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **101:**5048–5052.
35. **Chim, H., B. H. Tan, and C. Song.** 2007. Five-year review of infections in a burn intensive care unit: high incidence of *Acinetobacter baumannii* in a tropical climate. *Burns* **33:**1008–1014.
36. **Cho, T., H. Hamatake, H. Kaminishi, Y. Hagihara, and K. Watanabe.** 1992. The relationship between cyclic adenosine 3′,5′-monophosphate and morphology in exponential phase *Candida albicans*. *Med. Mycol.* **30:**35–42.
37. **Chotirmall, S. H., E. O'Donoghue, K. Bennett, C. Gunaratnam, S. J. O'Neill, and N. G. McElvaney.** 2010. Sputum *Candida albicans* presages FEV1 decline and hospitalized exacerbations in cystic fibrosis. *Chest* **138:** 1186–1195.
38. **Coco, B. J., J. Bagg, L. J. Cross, A. Jose, J. Cross, and G. Ramage.** 2008. Mixed *Candida albicans* and *Candida glabrata* populations associated with the pathogenesis of denture stomatitis. *Oral Microbiol. Immunol.* **23:**377–383.
39. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. *Annu. Rev. Microbiol.* **49:**711–745.
40. **Coudeyras, S., G. Jugie, M. Vermerie, and C. Forestier.** 2008. Adhesion of human probiotic *Lactobacillus rhamnosus* to cervical and vaginal cells and interaction with vaginosis-associated pathogens. *Infect. Dis. Obstet. Gynecol.* **2008:**549640.
41. **Csank, C., and K. Haynes.** 2000. *Candida glabrata* displays pseudohyphal growth. *FEMS Microbiol. Lett.* **189:** 115–120.
42. **Cugini, C., M. W. Calfee, J. M. Farrow III, D. K. Morales, E. C. Pesci, and D. A. Hogan.** 2007. Farnesol, a common sesquiterpene, inhibits PQS production in *Pseudomonas aeruginosa*. *Mol. Microbiol.* **65:**896–906.
42a. **Cugini, C., D. K. Morales, and D. A. Hogan.** 2010. *Candida albicans*-produced farnesol stimulates *Pseudomonas quinolone* signal production in LasR-defective *Pseudomonas aeruginosa* strains. *Microbiology* **156**: 3096–3107.
43. **Cugini, C., R. Kolter, and D. A. Hogan.** 2008. Interdomain cross talk, p. 419–429. *In* S. C. Winans and B. L. Bassler (ed.), *Chemical Communications among Bacteria*. ASM Press, Washington, DC.
44. **Cushion, M. T., M. S. Collins, and M. J. Linke.** 2009. Biofilm formation by *Pneumocystis* spp. *Eukaryot. Cell* **8:**197–206.
45. **Davis-Hanna, A., A. E. Piispanen, L. I. Stateva, and D. A. Hogan.** 2008. Farnesol and dodecanol effects on the *Candida albicans* Ras1-camp signalling pathway and the regulation of morphogenesis. *Mol. Microbiol.* **67:**47–62.
46. **de Carvalho, F. G., D. S. Silva, J. Hebling, L. C. Spolidorio, and D. M. P. Spolidorio.** 2006. Presence of mutans streptococci and *Candida* spp. in dental plaque/dentine of carious teeth and early childhood caries. *Arch. Oral Biol.* **51:**1024–1028.
47. **de Macedo, J. L., and J. B. Santos.** 2005. Bacterial and fungal colonization of burn wounds. *Mem. Inst. Oswaldo Cruz* **100:**535–539.
48. **Derengowski, L., C. De-Souza-Silva, S. Braz, T. Mello-De-Sousa, S. Bao, C. Kyaw, and I. Silva-Pereira.** 2009. Antimicrobial effect of farnesol, a *Candida albicans* quorum sensing molecule, on *Paracoccidioides brasiliensis* growth and morphogenesis. *Ann. Clin. Microbiol. Antimicrob.* **8:**13.
49. **Deveau, A., A. E. Piispanen, A. A. Jackson, and D. A. Hogan.** 2010. Farnesol induces hydrogen peroxide resistance in *Candida albicans* yeast by inhibiting the Ras-cyclic AMP signaling pathway. *Eukaryot. Cell* **9:**569–577.
50. **Dichtl, K., F. Ebel, F. Dirr, F. H. Routier, J. Heesemann, and J. Wagener.** 2010. Farnesol misplaces tip-localized Rho proteins and inhibits cell wall integrity signalling in *Aspergillus fumigatus*. *Mol. Microbiol.* **76:**1191–1204.
51. **Dieterich, C., M. Schandar, M. Noll, F. J. Johannes, H. Brunner, T. Graeve, and S. Rupp.** 2002. *In vitro* reconstructed human epithelia reveal contributions of *Candida albicans EFG1* and *CPH1* to adhesion and invasion. *Microbiology* **148:**497–506.
52. **Dyess, D. L., R. N. Garrison, and D. E. Fry.** 1985. *Candida* sepsis: implications of polymicrobial blood-borne infection. *Arch. Surg.* **120:**345–348.
53. **Ehrhardt, A., G. R. Ehrhardt, X. Guo, and J. W. Schrader.** 2002. Ras and relatives—job sharing and networking keep an old family together. *Exp. Hematol.* **30:**1089–1106.
54. **Elahi, S., G. Pang, R. Ashman, and R. Clancy.** 2005. Enhanced clearance of *Candida albicans* from the oral cavities of mice following oral administration of *Lactobacillus acidophilus*. *Clin. Exp. Immunol.* **141:**29–36.
55. **El-Azizi, M. A., S. E. Starks, and N. Khardori.** 2004. Interactions of *Candida albicans* with other *Candida* spp. and bacteria in the biofilms. *J. Appl. Microbiol.* **96:** 1067–1073.
56. **Elving, G., H. van der Mei, H. Busscher, R. van Weissenbruch, and F. Albers.** 2003. Influence of different combinations of bacteria and yeasts in voice prosthesis biofilms on air flow resistance. *Antonie van Leeuwenhoek* **83:**45–55.
57. **Elving, G. J., H. C. van der Mei, H. J. Busscher, R. van Weissenbruch, and F. W. J. Albers.** 2001. Air-flow resistances of silicone rubber voice prostheses after formation of bacterial and fungal biofilms. *J. Biomed. Materials Res.* **58:** 421–426.
58. **Eubanks, P. J., C. de Virgilio, S. Klein, and F. Bongard.** 1993. *Candida* sepsis in surgical patients. *Am. J. Surg.* **166:**617–619; discussion, 619–620.
59. **Evans, M. E., and M. Pollack.** 1993. Effect of antibiotic class and concentration on the release of lipopolysaccharide from *Escherichia coli*. *J. Infect. Dis.* **167:**1336–1343.
60. **Falagas, M. E., G. I. Betsi, and S. Athanasiou.** 2006. Probiotics for prevention of recurrent vulvovaginal candidiasis: a review. *J. Antimicrob. Chemother.* **58:**266–272.
61. **Fang, H. M., and Y. Wang.** 2006. RA domain-mediated interaction of Cdc35 with Ras1 is essential for increasing cellular cAMP level for *Candida albicans* hyphal development. *Mol. Microbiol.* **61:**484–496.
62. **Feng, Q., E. Summers, B. Guo, and G. Fink.** 1999. Ras signaling is required for serum-induced hyphal differentiation in *Candida albicans*. *J. Bacteriol.* **181:**6339–6346.
63. **Fitzsimmons, N., and D. R. Berry.** 1994. Inhibition of *Candida albicans* by *Lactobacillus acidophilus*: evidence for

the involvement of a peroxidase system. *Microbios* **80:** 125–133.
64. **Gaddy, J. A., A. P. Tomaras, and L. A. Actis.** 2009. The *Acinetobacter baumannii* 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and in the interaction of this pathogen with eukaryotic cells. *Infect. Immun.* **77:**3150–3160.
65. **Gale, C., D. Finkel, N. Tao, M. Meinke, M. McClellan, J. Olson, K. Kendrick, and M. Hostetter.** 1996. Cloning and expression of a gene encoding an integrin-like protein in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **93:** 357–361.
66. **Gibson, J., A. Sood, and D. A. Hogan.** 2009. *Pseudomonas aeruginosa-Candida albicans* interactions: localization and fungal toxicity of a phenazine derivative. *Appl. Environ. Microbiol.* **75:**504–513.
67. **Hall-Stoodley, L., J. W. Costerton, and P. Stoodley.** 2004. Bacterial biofilms: from the natural environment to infectious diseases. *Nat. Rev. Microbiol.* **2:**95–108.
68. **Han, Y. W., W. Shi, G. T. Huang, S. Kinder Haake, N. H. Park, H. Kuramitsu, and R. J. Genco.** 2000. Interactions between periodontal bacteria and human oral epithelial cells: *Fusobacterium nucleatum* adheres to and invades epithelial cells. *Infect. Immun.* **68:**3140–3146.
69. **Harcus, D., A. Nantel, A. Marcil, T. Rigby, and M. Whiteway.** 2004. Transcription profiling of cyclic AMP signaling in *Candida albicans*. *Mol. Biol. Cell* **15:**4490–4499.
70. **Harriott, M. M., and M. C. Noverr.** 2009. *Candida albicans* and *Staphylococcus aureus* form polymicrobial biofilms: effects on antimicrobial resistance. *Antimicrob. Agents Chemother.* **53:**3914–3922.
71. **Hawser, S. P., and L. J. Douglas.** 1994. Biofilm formation by *Candida* species on the surface of catheter materials in vitro. *Infect. Immun.* **62:**915–921.
72. **Hazan, R., J. He, G. Xiao, V. Dekimpe, Y. Apidianakis, B. Lesic, C. Astrakas, E. Deziel, F. Lepine, and L. G. Rahme.** 2010. Homeostatic interplay between bacterial cell-cell signaling and iron in virulence. *PLoS Pathog.* **6:**e1000810.
73. **Hermann, C., J. Hermann, U. Munzel, and R. Rüchel.** 1999. Bacterial flora accompanying *Candida* yeasts in clinical specimens. *Mycoses* **42:**619–627.
74. **Hess, D. J., M. J. Henry-Stanley, C. M. Bendel, B. Zhang, M.-A. Johnson, and C. L. Wells.** 2009. *Escherichia coli* and TNF-α modulate macrophage phagocytosis of *Candida glabrata*. *J. Surg. Res.* **155:**217–224.
75. **Hockey, L. J., N. K. Fujita, T. R. Gibson, D. Rotrosen, J. Z. Montgomerie, and J. E. Edwards, Jr.** 1982. Detection of fungemia obscured by concomitant bacteremia: in vitro and in vivo studies. *J. Clin. Microbiol.* **16:**1080–1085.
76. **Hoffman, L. R., H. D. Kulasekara, J. Emerson, L. S. Houston, J. L. Burns, B. W. Ramsey, and S. I. Miller.** 2009. *Pseudomonas aeruginosa lasR* mutants are associated with cystic fibrosis lung disease progression. *J. Cystic Fibrosis* **8:**66–70.
77. **Hogan, D. A., and R. Kolter.** 2002. *Pseudomonas-Candida* interactions: an ecological role for virulence factors. *Science* **296:**2229–2232.
78. **Hogan, D. A., Å. Vik, and R. Kolter.** 2004. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. *Mol. Microbiol.* **54:**1212–1223.
79. **Hogenauer, C., H. F. Hammer, G. J. Krejs, and E. C. Reisinger.** 1998. Mechanisms and management of antibiotic-associated diarrhea. *Clin. Infect. Dis.* **27:**702–710.
80. **Holmes, A. R., P. K. Gopal, and H. F. Jenkinson.** 1995. Adherence of *Candida albicans* to a cell surface polysaccharide receptor on *Streptococcus gordonii*. *Infect. Immun.* **63:**1827–1834.
81. **Holmes, A. R., R. McNab, and H. F. Jenkinson.** 1996. *Candida albicans* binding to the oral bacterium *Streptococcus gordonii* involves multiple adhesin-receptor interactions. *Infect. Immun.* **64:**4680–4685.
82. **Hornby, J. M., E. C. Jensen, A. D. Lisec, J. J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, and K. W. Nickerson.** 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. *Appl. Environ. Microbiol.* **67:**2982–2992.
83. **Hornby, J. M., and K. W. Nickerson.** 2004. Enhanced production of farnesol by *Candida albicans* treated with four azoles. *Antimicrob. Agents Chemother.* **48:**2305–2307.
84. **Hughes, W. T., and H. K. Kim.** 1973. Mycoflora in cystic fibrosis: some ecological aspects of *Pseudomonas aeruginosa* and *Candida albicans*. *Mycopathol. Mycol. Appl.* **50:**261–269.
85. **Inoue, Y., A. Shiraishi, T. Hada, K. Hirose, H. Hamashima, and J. Shimada.** 2004. The antibacterial effects of terpene alcohols on *Staphylococcus aureus* and their mode of action. *FEMS Microbiol. Lett.* **237:**325–331.
86. **Jabra-Rizk, M. A., W. A. Falkler, Jr., W. G. Merz, J. I. Kelley, A. A. M. A. Baqui, and T. F. Meiller.** 1999. Coaggregation of *Candida dubliniensis* with *Fusobacterium nucleatum*. *J. Clin. Microbiol.* **37:**1464–1468.
87. **Jabra-Rizk, M. A., T. F. Meiller, C. E. James, and M. E. Shirtliff.** 2006. Effect of farnesol on *Staphylococcus aureus* biofilm formation and antimicrobial susceptibility. *Antimicrob. Agents Chemother.* **50:**1463–1469.
88. **Jain, P., I. Akula, and T. Edlind.** 2003. Cyclic AMP signaling pathway modulates susceptibility of *Candida* species and *Saccharomyces cerevisiae* to antifungal azoles and other sterol biosynthesis inhibitors. *Antimicrob. Agents Chemother.* **47:**3195–3201.
89. **Jamieson, D. J., D. W. Stephen, and E. C. Terriere.** 1996. Analysis of the adaptive oxidative stress response of *Candida albicans*. *FEMS Microbiol. Lett.* **138:**83–88.
90. **Jenkinson, H. F., H. C. Lala, and M. G. Shepherd.** 1990. Coaggregation of *Streptococcus sanguis* and other streptococci with *Candida albicans*. *Infect. Immun.* **58:** 1429–1436.
91. **Kadosh, D., and A. D. Johnson.** 2005. Induction of the *Candida albicans* filamentous growth program by relief of transcriptional repression: a genome-wide analysis. *Mol. Biol. Cell* **16:**2903–2912.
92. **Kaewsrichan, J., K. Peeyananjarassri, and J. Kongprasertkit.** 2006. Selection and identification of anaerobic lactobacilli producing inhibitory compounds against vaginal pathogens. *FEMS Immunol. Med. Microbiol.* **48:**75–83.
93. **Kamaguchi, A., H. Baba, M. Hoshi, and K. Inomata.** 1994. Coaggregation between *Porphyromonas gingivalis* and mutans streptococci. *Microbiol. Immunol.* **38:**457–460.
94. **Kamaguchi, A., K. Nakayama, S. Ichiyama, R. Nakamura, T. Watanabe, M. Ohta, H. Baba, and T. Ohyama.** 2003. Effect of *Porphyromonas gingivalis* vesicles on coaggregation of *Staphylococcus aureus* to oral microorganisms. *Curr. Microbiol.* **47:**485–491.
95. **Kerr, J. R.** 1994. Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. *J. Clin. Microbiol.* **32:** 525–527.
96. **Klaerner, H. G., M. E. Uknis, R. D. Acton, P. S. Dahlberg, C. Carlone-Jambor, and D. L. Dunn.** 1997. *Candida albicans* and *Escherichia coli* are synergistic pathogens during experimental microbial peritonitis. *J. Surg. Res.* **70:**161–165.
97. **Klotz, S. A., B. S. Chasin, B. Powell, N. K. Gaur, and P. N. Lipke.** 2007. Polymicrobial bloodstream infections involving *Candida* species: analysis of patients and review of the literature. *Diagn. Microbiol. Infect. Dis.* **59:**401–406.

98. **Klotz, S. A., N. K. Gaur, R. De Armond, D. Sheppard, N. Khardori, J. E. Edwards, P. N. Lipke, and M. El-Azizi.** 2007. *Candida albicans* Als proteins mediate aggregation with bacteria and yeasts. *Med. Mycol.* 45:363–370.
99. **Krom, B. P., K. Buijssen, H. J. Busscher, and H. C. van der Mei.** 2009. *Candida* biofilm analysis in the artificial throat using FISH. *Methods Mol. Biol.* **499:**45–54.
100. **Kruppa, M., B. P. Krom, N. Chauhan, A. V. Bambach, R. L. Cihlar, and R. A. Calderone.** 2004. The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. *Eukaryot. Cell* **3:**1062–1065.
101. **Kuroda, M., S. Nagasaki, and T. Ohta.** 2007. Sesquiterpene farnesol inhibits recycling of the c55 lipid carrier of the murein monomer precursor contributing to increased susceptibility to β-lactams in methicillin-resistant *Staphylococcus aureus*. *J. Antimicrob. Chemother.* **59:**425–432.
102. **Labrousse, A., S. Chauvet, C. Couillault, C. L. Kurz, and J. J. Ewbank.** 2000. *Caenorhabditis elegans* is a model host for *Salmonella typhimurium*. *Curr. Biol.* **10:** 1543–1545.
103. **Lamont, R. J., and H. F. Jenkinson.** 1998. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiol. Mol. Biol. Rev.* **62:**1244–1263.
104. **Langford, M. L., A. L. Atkin, and K. W. Nickerson.** 2009. Cellular interactions of farnesol, a quorum-sensing molecule produced by *Candida albicans*. *Future Microbiol.* **4:**1353–1362.
105. **Langford, M. L., S. Hasim, K. W. Nickerson, and A. L. Atkin.** 2010. Activity and toxicity of farnesol towards *Candida albicans* are dependent on growth conditions. *Antimicrob. Agents Chemother.* **54:**940–942.
106. **Leberer, E., D. Harcus, D. Dignard, L. Johnson, S. Ushinsky, D. Y. Thomas, and K. Schroppel.** 2001. Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*. *Mol. Microbiol.* **42:** 673–687.
107. **Leunisse, C., R. van Weissenbruch, H. J. Busscher, H. C. van der Mei, and F. W. Albers.** 1999. The artificial throat: a new method for standardization of *in vitro* experiments with tracheo-oesophageal voice prostheses. *Acta Otolaryngol.* **119:**604–608.
108. **Li, D., D. Williams, D. Lowman, M. A. Monteiro, X. Tan, M. Kruppa, W. Fonzi, E. Roman, J. Pla, and R. Calderone.** 2009. The *Candida albicans* histidine kinase Chk1p: signaling and cell wall mannan. *Fungal Genet. Biol.* **46:**731–741.
109. **Lo, H. J., J. R. Kohler, B. DiDomenico, D. Loebenberg, A. Cacciapuoti, and G. R. Fink.** 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90:**939–949.
110. **Lorenz, M. C., J. A. Bender, and G. R. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot. Cell* **3:**1076–1087.
111. **Machida, K., and T. Tanaka.** 1999. Farnesol-induced generation of reactive oxygen species dependent on mitochondrial transmembrane potential hyperpolarization mediated by F_0F_1-ATPase in yeast. *FEBS Lett.* **462:**108–112.
112. **Machida, K., T. Tanaka, K.-I. Fujita, and M. Taniguchi.** 1998. Farnesol-induced generation of reactive oxygen species via indirect inhibition of the mitochondrial electron transport chain in the yeast *Saccharomyces cerevisiae*. *J. Bacteriol.* **180:**4460–4465.
113. **Maidan, M. M., L. De Rop, J. Serneels, S. Exler, S. Rupp, H. Tournu, J. M. Thevelein, and P. Van Dijck.** 2005. The G protein-coupled receptor Gpr1 and the Gα protein Gpa2 act through the cAMP-protein kinase A pathway to induce morphogenesis in *Candida albicans*. *Mol. Biol. Cell* **16:**1971–1986.
114. **Makoto, K., N. Sanae, I. Ryuta, and O. Toshiko.** 2007. Sesquiterpene farnesol as a competitive inhibitor of lipase activity of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* **273:**28–34.
115. **Martins, M., M. Henriques, J. Azeredo, S. M. Rocha, M. A. Coimbra, and R. Oliveira.** 2007. Morphogenesis control in *Candida albicans* and *Candida dubliniensis* through signaling molecules produced by planktonic and biofilm cells. *Eukaryot. Cell* **6:**2429–2436.
116. **McAlester, G., F. O'Gara, and J. P. Morrissey.** 2008. Signal-mediated interactions between *Pseudomonas aeruginosa* and *Candida albicans*. *J. Med. Microbiol.* **57:**563–569.
117. **McNab, R., A. R. Holmes, J. M. Clarke, G. W. Tannock, and H. F. Jenkinson.** 1996. Cell surface polypeptide CshA mediates binding of *Streptococcus gordonii* to other oral bacteria and to immobilized fibronectin. *Infect. Immun.* **64:**4204–4210.
118. **Mitchell, A. P.** 1998. Dimorphism and virulence in *Candida albicans*. *Curr. Opin. Microbiol.* **1:**687–692.
118a. **Morales, D. K., N. J. Jacobs, S. Rajamani, M. Krishnamurthy, J. C. Cubillos-Ruiz, and D. A. Hogan.** 2010. Antifungal mechanisms by which a novel *Pseudomonas aeruginosa* phenazine toxin kills *Candida albicans* in biofilms. *Mol. Microbiol.* **78:**1379–1392.
119. **Moran, G. P., D. J. Sullivan, M. C. Henman, C. E. McCreary, B. J. Harrington, D. B. Shanley, and D. C. Coleman.** 1997. Antifungal drug susceptibilities of oral *Candida dubliniensis* isolates from human immunodeficiency virus (HIV)-infected and non-HIV-infected subjects and generation of stable fluconazole-resistant derivatives in vitro. *Antimicrob. Agents Chemother.* **41:**617–623.
120. **Morris, A., K. Wei, K. Afshar, and L. Huang.** 2008. Epidemiology and clinical significance of *Pneumocystis* colonization. *J. Infect. Dis.* **197:**10–17.
121. **Navarathna, D. H. M. L. P., J. M. Hornby, N. Krishnan, A. Parkhurst, G. E. Duhamel, and K. W. Nickerson.** 2007. Effect of farnesol on a mouse model of systemic candidiasis, determined by use of a *dpp3* knockout mutant of *Candida albicans*. *Infect. Immun.* **75:**1609–1618.
122. **Nobbs, A. H., B. H. Shearer, M. Drobni, M. A. Jepson, and H. F. Jenkinson.** 2007. Adherence and internalization of *Streptococcus gordonii* by epithelial cells involves beta1 integrin recognition by SspA and SspB (antigen I/II family) polypeptides. *Cell. Microbiol.* **9:**65–83.
123. **Noverr, M. C., and G. B. Huffnagle.** 2004. Regulation of *Candida albicans* morphogenesis by fatty acid metabolites. *Infect. Immun.* **72:**6206–6210.
124. **Nseir, S., E. Jozefowicz, B. Cavestri, B. Sendid, C. Di Pompeo, F. Dewavrin, R. Favory, M. Roussel-Delvallez, and A. Durocher.** 2007. Impact of antifungal treatment on *Candida-Pseudomonas* interaction: a preliminary retrospective case-control study. *Intensive Care Med.* **33:**137–142.
125. **Nuzzo, I., M. R. Sanges, A. Folgore, and C. R. Carratelli.** 2000. Apoptosis of human keratinocytes after bacterial invasion. *FEMS Immunol. Med. Microbiol.* **27:** 235–240.
126. **Oh, K.-B., H. Miyazawa, T. Naito, and H. Matsuoka.** 2001. Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **98:**4664–4668.
127. **O'Sullivan, J. M., R. D. Cannon, P. A. Sullivan, and H. F. Jenkinson.** 1997. Identification of salivary basic proline-rich proteins as receptors for *Candida albicans* adhesion. *Microbiology* **143:**341–348.

128. **O'Sullivan, J. M., H. F. Jenkinson, and R. D. Cannon.** 2000. Adhesion of *Candida albicans* to oral streptococci is promoted by selective adsorption of salivary proteins to the streptococcal cell surface. *Microbiology* **146:**41–48.
129. **Paster, B. J., S. K. Boches, J. L. Galvin, R. E. Ericson, C. N. Lau, V. A. Levanos, A. Sahasrabudhe, and F. E. Dewhirst.** 2001. Bacterial diversity in human subgingival plaque. *J. Bacteriol.* **183:**3770–3783.
130. **Paulitsch, A., W. Weger, G. Ginter-Hanselmayer, E. Marth, and W. Buzina.** 2006. A 5-year (2000–2004) epidemiological survey of *Candida* and non-*Candida* yeast species causing vulvovaginal candidiasis in Graz, Austria. *Mycoses* **49:**471–475.
131. **Peleg, A. Y., D. A. Hogan, and E. Mylonakis.** 2010. Medically important bacterial-fungal interactions. *Nat. Rev. Microbiol.* **8:**340–349.
132. **Peleg, A. Y., E. Tampakakis, B. B. Fuchs, G. M. Eliopoulos, R. C. Moellering, and E. Mylonakis.** 2008. Prokaryote-eukaryote interactions identified by using *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **105:** 14585–14590.
133. **Pereira-Cenci, T., D. M. Deng, E. A. Kraneveld, E. M. M. Manders, A. A. Del Bel Cury, J. M. ten Cate, and W. Crielaard.** 2008. The effect of *Streptococcus mutans* and *Candida glabrata* on *Candida albicans* biofilms formed on different surfaces. *Arch. Oral Biol.* **53:**755–764.
134. **Peres-Bota, D., H. Rodriguez-Villalobos, G. Dimopoulos, C. Melot, and J. L. Vincent.** 2004. Potential risk factors for infection with *Candida* spp. in critically ill patients. *Clin. Microbiol. Infect.* **10:**550–555.
135. **Pfaller, M. A.** 1996. Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. *Clin. Infect. Dis.* **22**(Suppl. 2)**:**S89–S94.
136. **Pfaller, M. A., and D. J. Diekema.** 2010. Epidemiology of invasive mycoses in North America. *Crit. Rev. Microbiol.* **36:**1–53.
137. **Ponniah, G., C. Rollenhagen, Y. S. Bahn, J. F. Staab, and P. Sundstrom.** 2007. State of differentiation defines buccal epithelial cell affinity for cross-linking to *Candida albicans* Hwp1. *J. Oral Pathol. Med.* **36:**456–467.
138. **Pulimood, S., L. Ganesan, G. Alangaden, and P. Chandrasekar.** 2002. Polymicrobial candidemia. *Diagn. Microbiol. Infect. Dis.* **44:**353–357.
139. **Raad, I. I., and H. A. Hanna.** 2002. Intravascular catheter-related infections: new horizons and recent advances. *Arch. Intern. Med.* **162:**871–878.
140. **Ramage, G., S. P. Saville, B. L. Wickes, and J. L. Lopez-Ribot.** 2002. Inhibition of *Candida albicans* biofilm formation by farnesol, a quorum-sensing molecule. *Appl. Environ. Microbiol.* **68:**5459–5463.
141. **Ramage, G., K. VandeWalle, J. L. Lopez-Ribot, and B. L. Wickes.** 2002. The filamentation pathway controlled by the Efg1 regulator protein is required for normal biofilm formation and development in *Candida albicans*. *FEMS Microbiol. Lett.* **214:**95–100.
142. **Rauceo, J. M., R. De Armond, H. Otoo, P. C. Kahn, S. A. Klotz, N. K. Gaur, and P. N. Lipke.** 2006.Threonine-rich repeats increase fibronectin binding in the *Candida albicans* adhesin Als5p. *Eukaryot. Cell* **5:**1664–1673.
143. **Reeves, E. P., H. Lu, H. L. Jacobs, C. G. M. Messina, S. Bolsover, G. Gabella, E. O. Potma, A. Warley, J. Roes, and A. W. Segal.** 2002. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature* **416:**291–297.
144. **Richards, M. J., J. R. Edwards, D. H. Culver, and R. P. Gaynes.** 1999. Nosocomial infections in medical intensive care units in the United States. National Nosocomial Infections Surveillance System. *Crit. Care Med.* **27:** 887–892.
145. **Román, E., R. Alonso-Monge, Q. Gong, D. Li, R. Calderone, and J. Pla.** 2009. The cek1 MAPK is a short-lived protein regulated by quorum sensing in the fungal pathogen *Candida albicans*. *FEMS Yeast Res.* **9:**942–955.
146. **Ronnqvist, D., U. Forsgren-Brusk, U. Husmark, and E. Grahn-Hakansson.** 2007. *Lactobacillus fermentum* Ess-1 with unique growth inhibition of vulvo-vaginal candidiasis pathogens. *J. Med. Microbiol.* **56:**1500–1504.
147. **Rosenthal, V. D., D. G. Maki, R. Salomao, C. A. Moreno, Y. Mehta, F. Higuera, L. E. Cuellar, O. A. Arikan, R. Abouqal, and H. Leblebicioglu.** 2006. Device-associated nosocomial infections in 55 intensive care units of 8 developing countries. *Ann. Intern. Med.* **145:** 582–591.
148. **Rossignol, T., M. E. Logue, K. Reynolds, M. Grenon, N. F. Lowndes, and G. Butler.** 2007. Transcriptional response of *Candida parapsilosis* following exposure to farnesol. *Antimicrob. Agents Chemother.* **51:**2304–2312.
149. **Roux, D., S. Gaudry, D. Dreyfuss, J. El-Benna, N. de Prost, E. Denamur, G. Saumon, and J.-D. Ricard.** 2009. *Candida albicans* impairs macrophage function and facilitates *Pseudomonas aeruginosa* pneumonia in rat. *Crit. Care Med.* **37:**1062–1067.
150. **Saville, S. P., A. L. Lazzell, C. Monteagudo, and J. L. Lopez-Ribot.** 2003. Engineered control of cell morphology in vivo reveals distinct roles for yeast and filamentous forms of *Candida albicans* during infection. *Eukaryot. Cell* **2:**1053–1060.
151. **Semighini, C. P., J. M. Hornby, R. Dumitru, K. W. Nickerson, and S. D. Harris.** 2006. Farnesol-induced apoptosis in *Aspergillus nidulans* reveals a possible mechanism for antagonistic interactions between fungi. *Mol. Microbiol.* **59:**753–764.
152. **Shirtliff, M. E., B. P. Krom, R. A. M. Meijering, B. M. Peters, J. Zhu, M. A. Scheper, M. L. Harris, and M. A. Jabra-Rizk.** 2009. Farnesol-induced apoptosis in *Candida albicans*. *Antimicrob. Agents Chemother.* 53: 2392–2401.
153. **Smith, D. A., S. Nicholls, B. A. Morgan, A. J. P. Brown, and J. Quinn.** 2004. A conserved stress-activated protein kinase regulates a core stress response in the human pathogen *Candida albicans*. *Mol. Biol. Cell* **15:**4179–4190.
154. **Spear, G. T., M. R. Zariffard, M. H. Cohen, and B. E. Sha.** 2008. Vaginal IL-8 levels are positively associated with *Candida albicans* and inversely with lactobacilli in HIV-infected women. *J. Reprod. Immunol.* **78:**76–79.
155. **Staab, J. F., S. D. Bradway, P. L. Fidel, and P. Sundstrom.** 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* **283:**1535–1538.
156. **Sullivan, D. J., G. P. Moran, E. Pinjon, A. Al-Mosaid, C. Stokes, C. Vaughan, and D. C. Coleman.** 2004. Comparison of the epidemiology, drug resistance mechanisms, and virulence of *Candida dubliniensis* and *Candida albicans*. *FEMS Yeast Res.* **4:**369–376.
157. **Tampakakis, E., A. Y. Peleg, and E. Mylonakis.** 2009. Interaction of *Candida albicans* with an intestinal pathogen, *Salmonella enterica* serovar Typhimurium. *Eukaryot. Cell* **8:**732–737.
158. **Thein, Z. M., Y. H. Samaranayake, and L. P. Samaranayake.** 2006. Effect of oral bacteria on growth and survival of *Candida albicans* biofilms. *Arch. Oral Biol.* **51:**672–680.
159. **Uppuluri, P., A. K. Chaturvedi, A. Srinivasan, M. Banerjee, A. K. Ramasubramaniam, J. R. Köhler, D. Kadosh, and J. L. Lopez-Ribot.** 2010. Dispersion as an important step in the *Candida albicans* biofilm developmental cycle. *PLoS Pathog.* **6:**e1000828.

160. **Uppuluri, P., S. Mekala, and W. L. Chaffin.** 2007. Farnesol-mediated inhibition of *Candida albicans* yeast growth and rescue by a diacylglycerol analogue. *Yeast* **24:**681–693.
161. **Verghese, A., K. Prabhu, D. R. Diamond, and A. Sugar.** 1988. Synchronous bacterial and fungal septicemia: a marker for the critically ill surgical patient. *Am. Surg.* **54:**276–283.
162. **Wagner, R. D., C. Pierson, T. Warner, M. Dohnalek, J. Farmer, L. Roberts, M. Hilty, and E. Balish.** 1997. Biotherapeutic effects of probiotic bacteria on candidiasis in immunodeficient mice. *Infect. Immun.* **65:**4165–4172.
163. **Wang, L.-H., Y. He, Y. Gao, J. E. Wu, Y.-H. Dong, C. He, S. X. Wang, L.-X. Weng, J.-L. Xu, L. Tay, R. X. Fang, and L.-H. Zhang.** 2004. A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Mol. Microbiol.* **51:**903–912.
164. **Weber, K., R. Sohr, B. Schulz, M. Fleischhacker, and M. Ruhnke.** 2008. Secretion of *E,E*-farnesol and biofilm formation in eight different *Candida* species. *Antimicrob. Agents Chemother.* **52:**1859–1861. (Erratum, **53:**848, 2009.)
165. **Westwater, C., E. Balish, and D. A. Schofield.** 2005. *Candida albicans*-conditioned medium protects yeast cells from oxidative stress: a possible link between quorum sensing and oxidative stress resistance. *Eukaryot. Cell* **4:** 1654–1661.
166. **Wilson, D., A. Tutulan-Cunita, W. Jung, N. C. Hauser, R. Hernandez, T. Williamson, K. Piekarska, S. Rupp, T. Young, and L. Stateva.** 2007. Deletion of the high-affinity cAMP phosphodiesterase encoded by *PDE2* affects stress responses and virulence in *Candida albicans*. *Mol. Microbiol.* **65:**841–856.
167. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317. (Erratum, **39:**1093; erratum, **40:**1077, 2005.)
168. **Xu, X.-L., R. T. H. Lee, H.-M. Fang, Y.-M. Wang, R. Li, H. Zou, Y. Zhu, and Y. Wang.** 2008. Bacterial peptidoglycan triggers *Candida albicans* hyphal growth by directly activating the adenylyl cyclase Cyr1p. *Cell Host Microbe* **4:**28–39.
169. **Zheng, X., Y. Wang, and Y. Wang.** 2004. Hgc1, a novel hypha-specific G1 cyclin-related protein, regulates *Candida albicans* hyphal morphogenesis. *EMBO J.* **23:** 1845–1856.

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch21

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Back to the Future: *Candida* Mitochondria and Energetics

DEEPU ALEX, RICHARD CALDERONE, AND DONGMEI LI

OVERVIEW

Mitochondria are the energy centers for eukaryotic cells, and as such, their functions extend to and include amino acid, lipid, and iron metabolism. They are associated with transporter functions, protein degradation, programmed cell death, signaling, cell division and growth, and numerous other processes. In this chapter, we review their role in human fungal pathogens, focusing upon *Candida albicans*, *Candida glabrata*, and *Candida parapsilosis* since, by far, studies with these three pathogens exceed others. The respiratory pathways of *Candida* species are highlighted along with the roles of mitochondria in morphogenesis, adaptive responses to oxidant stress and carbon depletion, and antifungal drug resistance and sensitivity. Aside from these roles, there is growing evidence that adaptation and change associated with carbon metabolism provide flexibility for survival of these pathogens in nutrient-starved niches of the human host. The focus therefore is on the role of mitochondria in the pathogenesis of candidiasis and developing the concept that there are differences in mitochondria of mammalian cells versus pathogens. The latter point serves to indicate that there is the possibility of antifungal drugs that target only fungal mitochondria. Finally, we leave the reader with the current understanding of how mitochondria manage to function by only synthesizing 1% of the ~1,000 proteins that are found there.

A HISTORICAL PERSPECTIVE

The study of mitochondria in *Candida* species first followed those that were of industrial importance, examples being *C. utilis* and *C. lipolytica* (29, 51, 61). These species were valued as either a flavoring addition to processed foods and single-cell protein or in the petroleum industry. Naturally, genetic engineering was applied to construct even better strains. But basic biological studies were also conducted to evaluate both primary and secondary metabolism, ultrastructure, and, of importance to the subject of this chapter, mitochondrial energetics and compartmentalization (7). Important discoveries were made along the way, including reports of a cyanide-insensitive respiration (51), the alternative oxidase (AOX) pathway, which was later shown to occur in *C. albicans* (59, 60). To understand the complexity of the respiratory pathways, inhibitors of specific electron transport chain (ETC) complexes (i.e., complexes I to V) were identified, such as, for example, rotenone (complex I), antimycin (complex III), cyanide and azide (complex IV), and the salicyl hydroxamic acid (SHAM) (AOX) pathway (41, 51). Mitochondrial mutants were identified as those unable to grow with nonfermentative carbon sources such as ethanol, acetate, and lactate but still able to utilize glucose. Within the past decade, especially, the model yeast has provided a wealth of information on protein translocation and sorting out of proteins to the outer membrane (OM), intermembrane space (IMS), inner membrane (IM), and matrix compartments of mitochondria, discussed below. The message from this review of mitochondria is that respiratory pathways as well as other proteins vital to mitochondrial functions are broadly conserved among plants, fungi, and mammals, yet there are major differences not only among diverse fungi but within groups of phylogenetically related fungi. These differences shed light on survival of fungal pathogens specifically and offer opportunities to develop ways to prevent disease from occurring.

RESPIRATION IN *CANDIDA* SPECIES

Mentioned above was the major effort of understanding industrial, nonpathogenic *Candida* species with regard to mitochondrial proteins and respiratory pathways. More recently, the design of mitochondrial respiratory pathways has included pathogenic *Candida* species such as *C. albicans* and *C. parapsilosis*. Below we summarize some of these important observations.

At least three mitochondrial respiratory pathways have been identified in these pathogens, depicted in Fig. 1 (9, 10, 19, 27, 30, 31, 38). They include the classical respiratory

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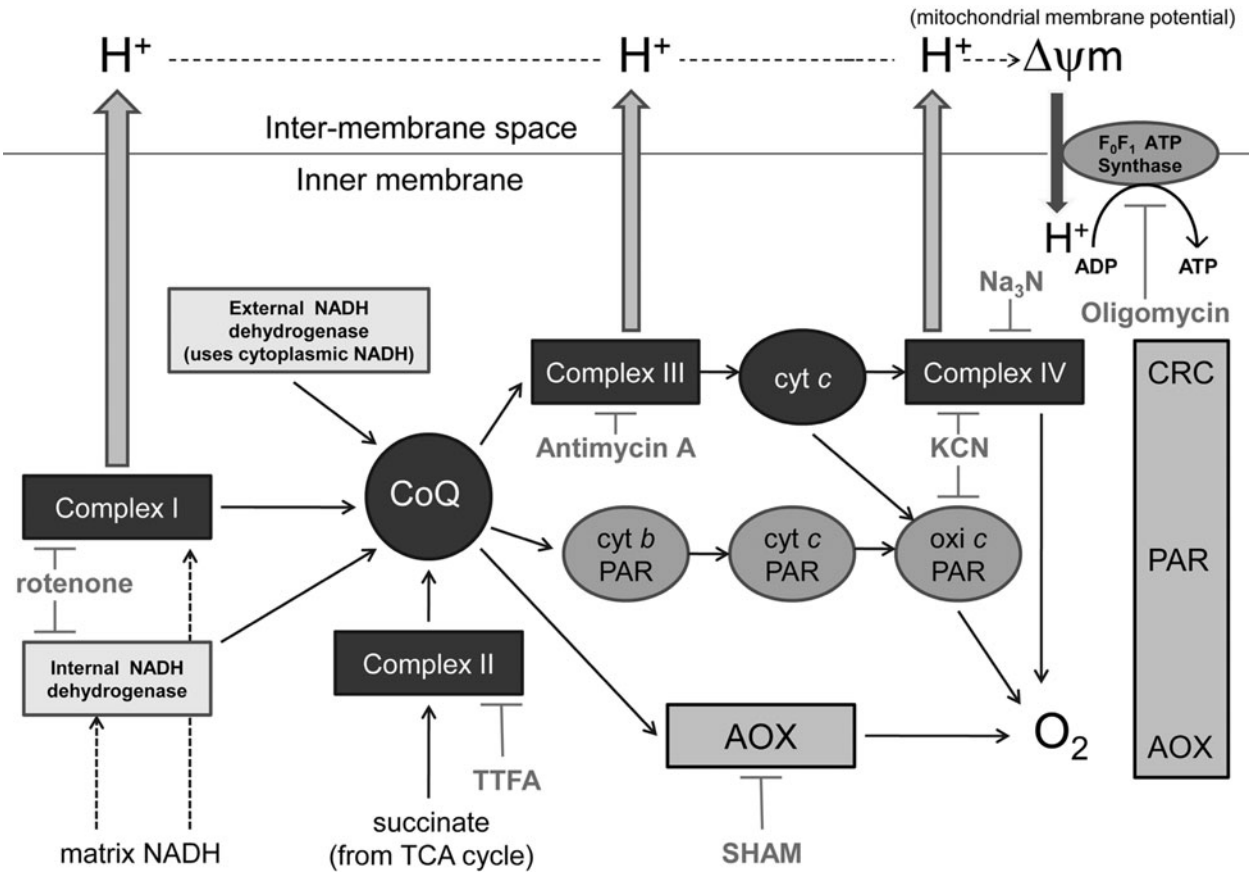

FIGURE 1 The respiratory pathway of *Candida albicans* and C. *parapsilosis*. Complexes I, II, III, and IV are part of the classical respiratory pathway. Protons are transported to the IMS as a result of electron transfer across complexes in the respiratory pathways, thereby building a potential across the membrane. CoQ is the hub for transfer of electrons from complex I and complex II. Additionally, there are alternative NADH dehydrogenases that can separately sequester NADH from internal and external sources and feed electrons to CoQ. The AOX pathway branches at the level of CoQ and is able to reduce oxygen and produce ATP. The PAR pathway also branches at the level of CoQ but is also able to accept electrons from cytochrome *c* (cyt *c*) of the classical pathway, which is further downstream of CoQ. Inhibitors of the complexes are shown. Of importance to energetics, the potential that is built up by the transfer of protons is used to generate ATP as the protons are pumped back into the matrix. oxi c, cytochrome oxidase. Adapted from reference 55. 10.1128/9781555817176.ch21f1

complex (CRC) pathway, the alternative oxidase (AOX) pathway, and the parallel respiratory (PAR) pathway. Internal NADH generated from an NADH mitochondrial dehydrogenase is processed through complex I, which consists of approximately 50 proteins. Complex I pumps H^+ to the IMS of mitochondria, resulting in the generation of a membrane potential (**ΔΨM**) which then is used together with an ATP synthase within the IM to make ATP. There are two other sites that also provide protons, complex III and complex IV. NADH is also provided by an external, cytoplasmic NADH dehydrogenase. Complex I functions through coenzyme Q (CoQ), the latter serving as an intermediate step of electron flow through complex III and complex IV. CoQ also provides electrons for the AOX and PAR pathways, discussed in more detail below. Complexes I, III, and IV as well as AOX inhibitors have provided a key to our understanding of the integration of the three pathways. Rotenone, antimycin A, and KCN inhibit complexes I, III, and IV, respectively, while SHAM is an inhibitor of the AOX pathway. The PAR pathway was described for C. *parapsilopsis* initially as a pathway distinct from the CRC and AOX pathways, since PAR is inhibited by KCN but not by antimycin A, the latter of which inhibits the CRC pathway (10, 27). What is the contribution of each of the routes for oxygen reduction (to water) using ADP/O ratios? This exquisite experiment was provided by Milani et al. (48). They show that the CRC capacity is about twice as large as that of AOX, while the PAR pathway is apparently used only when the AOX and CRC pathways are inhibited. Thus, the PAR pathway accounts for about one-tenth of the maximal oxygen consumption rate. The critical point of the PAR pathway is the flow of electrons from cytochrome *c* of the CRC to the PAR terminal oxidase, again highlighting the highly branched and flexible nature of respiration in this organism compared to vertebrate systems. C. *albicans* appears to operate its respiratory pathways in a manner similar to C. *parapsilosis*.

Importantly, the contribution of each pathway is not additive, as the AOX and PAR pathways appear more functionally important as compensatory systems. In contrast, the respiratory pathway in mammalian cells is more linear in the absence of AOX and PAR pathways. Other differences among vertebrates, model yeast, plants, and specific fungi with regard to the respiratory pathways are described in Table 1. As expected, *C. albicans* and *C. parapsilosis* are quite similar.

Single inhibitors or combinations of inhibitors led to the discovery of both the AOX and PAR pathways. For example, the AOX pathway is cyanide insensitive, while the combination of sodium azide and SHAM (interfering with the CRC and AOX pathways) still allowed mitochondrial respiration and the discovery of the PAR pathway (Fig. 1). It is clear that the majority of energy produced and oxygen consumed is due to the CRC pathway. However, a remarkable feature is the redundancy (compensation) that exists which allows those fungal pathogens (at least the ones studied) to still grow in the absence of, for example, both the CRC and AOX pathways, albeit not as rapidly. However, we should avoid thinking that the respiratory systems are totally conserved among mammals, model yeast, plants, and human-pathogenic fungi. In fact, human mitochondria lack an AOX pathway, and model yeast lacks complex I (Table 1). Also, as stated above, the vertebrate respiratory chain resembles a linear sequence, while fungal respiration is much more flexible and complex (56). Last but not least is the critical role that mitochondrial membrane potential (**ΔΨM**) plays in making ATP. But it is important to realize that the membrane potential generated by proton flow is only critical to inner mitochondrial membrane functions associated with proton flow generated along the electron transport system by complexes I, III, and IV. The OM of mitochondria is the gateway to the translocation cytoplasmic proteins and their distribution to the OM, IM, IMS, and inner matrix. Both translocation of cytoplasmic proteins and membrane potential are discussed below in a section on translocases.

TABLE 1 Differences in respiratory pathways among organisms

	Respiratory pathway			
Organisms	CRC	AOX	PAR	Uncoupling proteins
Vertebrates	NADH dehydrogenase (C I) and succinate dehydrogenase (C[a] II) transfer electrons derived from the Krebs cycle to the CoQ (ubiquinone) pool. CoQ passes the electrons to cytochrome *c* (C III), which is then transferred to cytochrome oxidase (C IV)	Absent	Absent	UCP or thermogenin is a protein exclusive to brown adipocytes that functions as a proton transporter using the gradient generated by the respiratory chain to dissipate heat as a mechanism of nonshivering thermogenesis (51)
Candida parapsilosis	Presence of alternative NADH dehydrogenase (rotenone insensitive) (48)	AOX pathway is cyanide insensitive; respiration capacity of CRC is two times higher than AOX (48)	Electron flux deviated upstream of C III; however, can also transfer electrons from cytochrome c of CRC to terminal oxidase of PAR; induction of PAR when both CRC and AOX are inhibited; one-tenth of maximal respiration capacity (11, 48)	CpUCP identified in mitochondria depleted of free fatty acids; stimulated by linoleic acid, inhibited by GTP (36)
Candida albicans	Similar to *C. parapsilosis* (11)	Similar to *C. parapsilosis*; AOX is induced by inhibition of CRC (11)		Similar to *C. parapsilosis* (11)
Saccharomyces cerevisiae	Absence of C I; presence of alternative NADH dehydrogenase (38)	Absent	Absent	Not found
Plants	Presence of alternative NADH dehydrogenase (38)	Positive regulation by pyruvate; existence of high-activity monomeric and low-activity dimeric forms (38)	Absent	Plant UCPs identified that are involved in cellular stress response (64)
Inhibitors	C I: rotenone, piericidin C III: antimycin A C IV: sodium azide, cyanide	SHAM; BHAM	Amital, high concentrations of myxothiazol and cyanide	

[a]C, complex.

It is interesting to compare the glycolytic circuits of *Saccharomyces cerevisiae* with that of *C. albicans*. In the former, a unique dependence upon fermentation as a source of energy is exhibited; only when no fermentable carbon sources are available will cells switch to a respiratory metabolism. Thus, *S. cerevisiae* is referred to as a "Crabtree-positive" organism which utilizes a glucose repression of largely aerobic respiration, in contrast to others, like *C. albicans*, which is "Crabtree negative" and which also relies upon the oxidation of substrates via the mitochondrial tricarboxylic acid (TCA) cycle (3). Other differences have been described for the mitochondrial circuits and regulation by these two organisms. For example, iron availability for cytochrome heme synthesis varied in the *C. albicans* YFH1 versus *S. cerevisiae* YFH1-iron binding protein frataxin, as determined with mutants of each (58).

In the following section, we discuss the role of mitochondria in growth and morphogenesis, adaptation to stress in vitro, antifungal targets, and survival of *C. albicans* in the host.

MITOCHONDRIA DO MORE THAN PRODUCE ATP

Respiration and Morphogenesis in *C. albicans*

The morphogenesis of *C. albicans* has long been featured as critical to the establishment of candidiasis. Early studies focused upon nutritional conditions that favored each growth form (15). The relative importance of glycolysis versus aerobic respiration (mitochondria) in growth and morphogenesis (yeast to filaments) with metabolism has also been investigated but largely remains unanswered. Two landmark papers were published in 1974 in which correlations of filamentous and yeast growth with respiratory metabolism were suggested; these represent the first attempts at a correlation between filamentous and yeast growth and respiratory metabolism (43, 44). These authors showed that filamentous cultures had slower oxygen consumption, produced less CO_2 and more ethanol, and had reduced FADH levels than yeast cultures, all of which led to the interpretation that the yeast-to-hypha switch was associated with a change from an aerobic metabolism to one of fermentative metabolism. Further, oxidative phosphorylation was suppressed during filament growth, described as a Crabtree effect (43, 44). Other data on the association of a morphology-related respiratory metabolism have supported the observation of Land et al. The mRNAs of four glycolytic genes (*PYK1*, *ADH1*, *PGK1*, and *GPM1*) increased during exponential growth of yeast cells and then decreased to relatively low levels in the stationary phase, supporting a correlation of yeast growth with glycolysis (62, 63).

In contrast to that described above, (i) a filamentous-only mutant of *C. albicans* consumed more oxygen than a "normal" strain growing as yeast cells but was otherwise qualitatively similar for both strains (65). This observation may be taken with caution because of the otherwise unknown physiological status of the mutant. (ii) Maltose (glycolysis) supported a filamentous growth, while acetate (mitochondria) favored a yeast growth (66). (iii) A *C .albicans* mutant in which the NADH dehydrogenase complex I gene of the electron transport system (*NDH51*) was deleted grew as a yeast even in filament-inducing media (47). A mitochondrial deficiency was supposed based upon the inability of the mutant to grow in the presence of acetate, a substrate for mitochondrial respiration. (iv) In support of this observation, a *C. albicans* strain in which a pyruvate dehydrogenase complex protein X (*PDX1*) was deleted displayed a defect in filamentous growth. As this protein complex catalyzes the conversion of pyruvate to acetyl coenzyme A, which is necessary for mitochondrial respiration, Pdx1p was interpreted as a compensatory protein that was extraneous to the ETC yet important to oxidative respiration (64). (v) Recently, Bambach et al. identified *C. albicans GOA1*, which is required for optimal yeast growth, filamentation, and virulence (4). Goa1p is transported to mitochondria during oxidative stress and is essential for mitochondrial functions, as the *goa1*Δ null mutant is unable to grow on several nonfermentative carbon sources and has a defective membrane potential associated with reduced ATP formation. Also, the null mutant grew minimally on 10% serum, Spider, and synthetic low-ammonium dextrose (SLAD) agar media, all of which are known inducers of filamentous growth. (vi) As described above in the section on *Candida* respiratory pathways, these species are referred to as being Crabtree negative, opposite of *S. cerevisiae*, which is Crabtree positive (3). Thus, this observation is in contrast to the Crabtree-negative definition of *C. albicans* as described by Land and coworkers (43, 44).

It would appear, therefore, that morphogenesis is associated with both glycolysis and consumption of glucose through mitochondria by aerobic respiration, and is metabolically critical to the interconversion of growth forms. It is probably more reasonable to think about morphogenesis as requiring a highly integrated mix of numerous cell activities such as cell division, DNA and RNA synthesis, cell wall synthesis, and numerous other types of cell activities. Thus, a disturbance of one affects the output of a cell to grow.

Adaptation to Stress, Especially ROS

Mitochondria play an active role in detoxifying reactive oxidant species (ROS) produced during cell metabolism. This has been convincingly shown through the use of inhibitors of respiratory complexes (56). *C. albicans* cells cultured in the presence of antimycin A, SHAM, or KCN showed limited growth, while rotenone (complex I inhibitor) had very little effect. Oxygen consumption was severely reduced when intact cells or mitochondria were treated with antimycin A only, but either antimycin A or KCN compromised oxygen consumption of isolated mitochondria. In cells treated with antimycin A or BHAM (a SHAM-like compound), ROS accumulation increased significantly. These data indicate that complex III or AOX blockade drastically affects growth probably through ROS accumulation. A similar observation has been made in the *goa1*Δ mutant described earlier, which also accumulated ROS more so than a gene-reconstituted strain or parental cells (unpublished data). The key antioxidant Sod2p is localized in mitochondria of *C. albicans*, further pointing to the central role of this organelle in reducing stress caused by superoxides (46). More recent information links increased ROS production and an increase in membrane potential with deletion of CaLPF7817, an otherwise unknown gene of *C. albicans* (37).

An interesting concept of survival of *C. albicans* in the host has been proposed by Cheng et al. (16, 17). They recovered a strain that was passaged through murine spleens five times. The phenotypes of this strain included reduced colony size, poor growth on media lacking glucose, increased O_2 consumption, increased numbers of mitochondria, an intact ETC, and an uncoupling of oxidative phosphorylation. The strain was also resistant to killing by neutrophils

and macrophages and persisted in the kidneys of animals in numbers equal to those of the parental SC5314 strain. The suggestion by these authors was that the uncoupling of oxidative phosphorylation might provide a way for the organism to survive during host stress. These results are to be compared with those for other respiratory mutants of *C. albicans* that have major damage to their ETC and were unable to sustain an infection in mice (2).

Mitochondrial uncoupling has not been studied to any great extent in *C. albicans*, but a few observations have been published in the *S. cerevisiae* literature that focus upon stress responses. For example, adaptation to mild heat shock, in which HSP104 (heat shock protein) plays a prominent role, causes a hyperpolarization of the IM of mitochondria (55). The coordination of these events to the adaptation is not completely understood, but uncouplers of mitochondria prevent HSP104 synthesis in the adaptive response and also dissipate heat (52). Other references to uncouplers are listed in Table 1.

Are Mitochondria Targets of Antifungal Drugs, and Can They Be?

Whether mitochondria are targets of antifungal drugs is difficult to answer, since data may be interpreted as an effect that is secondary to the primary target. Also, the presumed conservative nature of mitochondrial proteins perhaps gives pause to drug discovery implications, although we have mentioned above differences among mitochondrial proteins and pathways in mammals versus *Candida* species. Nevertheless, we look at examples that suggest changes in mitochondrial activity after treatment with an antifungal drug by focusing on what is described for new compounds that have not found clinical usage yet as well as clinically relevant antifungal drugs. The AOX and PAR pathways are not found in mammalian cells, described above, and therefore qualify as targets for drug discovery (11, 33, 34). The mechanism of action of a putative therapeutic could induce, among other effects, increased ROS, loss of mitochondrial function, and the initiation of apoptosis that has been associated with avirulence, at least for *C. albicans*.

PLE

Plagiochin E (PLE) is produced by the liverwort *Marchantia polymorpha* (67–69). Actually, a number of potential antifungal compounds have been identified from species of liverwort (70). More recent data have suggested that PLE induces apoptosis in *C. albicans* through a metacaspase-dependent apoptotic pathway (69). However, prior to that study, PLE was believed to cause mitochondrial dysfunction by enhancing increasing mitochondrial membrane potential while decreasing ATP levels (69). The decrease in ATP levels was believed the result of an inhibition of mitochondrial dehydrogenases, although no specific complex was mentioned. It is likely that the two observations are related, as mitochondrial dysfunction is a component of apoptosis.

CTBT

7-Chlorotetrazolo[5,1-c]benzo[1,2,4]triazine (CTBT) increases the activity of several antimycotics, including flucytosine as well as azoles. The activity of the drug requires molecular oxygen. Using a library of *S. cerevisiae* mutants, CTBT was postulated to increase oxidant sensitivity that resulted in transcriptional changes of a variety of genes, including DNA repair and mitochondrial genes such as *SOD2* (6). The primary lesion induced by CTBT will require a temporal analysis of treated cells, although petite mutants were especially susceptible to drugs.

Histatin 5

The target of histatin 5, a human basic salivary peptide that has demonstrated antifungal activity, was initially shown to be the energized mitochondria (32, 40). This hypothesis was based upon changes in mitochondrial membrane potential as well as the colocalization of tagged histatin 5 with MitoTracker, a mitochondrial stain. In favor of a mitochondrial target for histatin 5, mitochondrial DNA, small-colony mutants were resistant to histatin 5 (28). Further observations on histatins and *Candida species* are described in chapter 6.

CAS

C. parapsilosis isolates often have reduced susceptibility to the antifungal caspofungin (CAS). The mechanism underlying the high MIC of CAS is not understood but is probably of more than one type. Nevertheless, three clinical isolates of this species, each of which had a mean MIC_{50} of 2 μg/ml for CAS, demonstrated an increased sensitivity when CAS was assayed with antimycin A and BHAM (14). Neither respiratory inhibitor was effective when used alone with CAS. These data indicate that inhibition of CRC complex III and AOX results in increased drug sensitivity.

Azoles

C. glabrata is innately resistant to fluconazole. To understand this observation, Kaur et al. examined a library of *C. glabrata* of 9,216 random insertion mutants (39). Increased susceptibility was shown in two mutants lacking ABC transporters, while a third mutant was defective in *RTG2*, which is associated with retrograde signaling from mitochondria to the nucleus. Of 24 other mutants with altered susceptibilities, representing a variety of cell functions, one set was defective in mitochondrial assembly and organization. Correspondingly, these mutants had very high levels of resistance to fluconazole due to increased expression of CDR1 and CDR2 pumps (57). In contrast, reversible switching of petite mutants to strains that grow normally resulted in increased susceptibility to fluconazole and a corresponding decrease in pump expression. Further, during in vitro growth of *C. glabrata* with large amounts of fluconzaole, decreased susceptibility (high-frequency azole resistance) to the drug occurs that is associated with upregulation of drug pumps, but high-frequency azole resistance also occurred in strains that lost mitochondria following treatment with ethidium bromide, even though cells were not exposed to fluconazole treatment (57). Bouchara et al. (8) have also described a *C. glabrata* mutant that grew as small colonies (strain 1084-S). This strain had greater sensitivity to amphotericin B but was resistant to ketoconazole and fluconazole. Further analysis demonstrated alterations in mitochondria that were equated with a petite mutation. Similarly, a petite mutant of *C. albcians* had decreased susceptibility to fluconazole and voriconazole that was associated with overexpression of the MDR1 efflux pump (16). Each of these studies demonstrate that loss of mitochondrial function, exhibited as mild to severe phenotypes, is associated with a drug-specific change and greater sensitivity to amphotericin but resistance to azoles.

The relationship between the AOX pathway of *C. albicans* and fluconazole sensitivity was evaluated by using inhibitors with or without fluconazole (70). Cyanide treatment (induction of AOX) decreased susceptibility, while

SHAM (inhibitor of AOX) increased susceptibility to fluconazole. The combination of SHAM and fluconazole synergized killing of the organism, likely through an accumulation of ROS. In *C. albicans*, ketoconazole strongly reduced the activity of cytochrome a_3 when added to purified mitochondria (61).

TMP-1363

C. albicans and *C. glabrata* respiration-deficient mutants but not wild-type strains were significantly more sensitive to photodynamic treatment with cationic porphyrin photosensitizer *meso*-tetra (*N*-methyl-4-pyridyl) porphine tetra tosylate (TMP-1363) (12). This observation suggests that intact mitochondria can be targeted through drug discovery to improve the efficacy through synergy with photodynamic-treatment-induced phototoxicity in *Candida*. Another active compound is baicalein, which is a flavone, Chinese herbal medicine that initiates apoptosis via mitochondria in cells of *C. albicans* (18).

Carbon Metabolism and Environmental Niches

A paradigm for *C. albicans* during infection or as it colonizes the host is that metabolic flexibility must be exhibited as the organism encounters different host niches. The hypothesis put forth is that environments, such as the bloodstream, versus within phagocytes, such as neutrophils or macrophages, are quite different with regard to nutrients at these sites and stress conditions. For example, glucose is apparently not limiting in the bloodstream but can be quite low in host phagocytic cells. Consequently, an important adaptive mechanism is for *C. albicans* to change from glycolysis while in glucose-rich niches to a nonglycolytic (nonfermentative) metabolism. That adaptation requires major changes in transcriptional regulation of genes that reflect the need of the organism to grow and survive. In the case of a carbon limitation, an idea that has come to fruition from a number of investigative groups is a shift from glycolytic genes to metabolic processes such as the glyoxylate bypass mechanism, associated with mitochondria, and gluconeogenesis, processes that conserve carbon and lead to glucose synthesis that, respectively, reflect the low-carbon status of neutrophils and macrophages (5, 23, 24, 45, 54). The following observations support this concept.

A *C. albicans*-infected macrophage cell line (J774A) was compared to cells grown in vitro in RPMI plus 10% serum for transcriptional analysis by microarray hybridization at 0 to 6 h. Metabolic processes were evaluated at 1 and 6 h after infection of macrophages (data expressed as a ratio of 6 h/1 h). That ratio was low for ribosomal and glycolytic processes at 1 h postinfection. These data suggest that protein synthesis and glycolysis are repressed shortly after phagocytosis but that by 6 h postinfection, these metabolic processes increased, during which escape from macrophages by germ tube formation occurred. Conversely, the glyoxylate cycle and gluconeogenesis processes were much higher at 1 h but lower than ribosomal or glycolysis at 6 h. The take-home message is that mitochondrial pathways were required for adaptation of the organism to low carbon during early stages of internalization of the organism, which, presumably, is synthesizing glucose and conserving the loss of CO_2 through the glyoxylate cycle and gluconeogenesis. That adaptation may restore carbon metabolism to allow germination of the organism to occur and escape from the macrophage by lysis of the phagocyte. Additional support for this hypothesis was provided from mutants that lacked *FOX2* and *FBP1*, both of which are critical to fatty acid β-oxidation and gluconeogenesis (54). Both the *fox2*Δ and *fbp1*Δ null mutants had attenuated virulence, again reflecting a need for other types of metabolic processes in addition to glycolysis.

Another approach to the study of carbon adaptation by *C. albicans* was the use of specific gene reporter constructed strains instead of measuring global transcriptional responses. Thus, Barelle et al. (5) used four strains, each with a different gene-green fluorescent protein tag to represent glycolysis (*PFK2* or *PYK1*) or TCA cycle/gluconeogenesis (*ICL1* or *PCK1*) pathways. In vitro reporter activity in a medium comparing 2% glucose to an amino acid medium (sole carbon source) indicated transcription of the glycolysis genes but minimal transcription of the other two genes. In neutrophils, *ICL1* (glyoxylate cycle) and *PCK1* were transcribed to a greater extent than the glycolysis genes. Interestingly, in kidney of infected mice, the transcription of the glycolytic genes was much greater than that of *PCK1* and *ICL1*; this difference could be correlated with virulence, although not absolutely.

While the concept of transcription of metabolic processes that are required for survival during nutrient (carbon) stress seems reasonably correct, the transcriptional regulators have only been partially described. One of these is *ACE2*, which was previously identified in *S. cerevisiae* for its role in daughter and mother cell separation (66). In *C. albicans*, deletion of *ACE2* affects several processes, including a reduction of glycolytic enzymes, while mitochondrial respiratory enzymes of the TCA cycle, oxidative phosphorylation, and ATP synthesis are increased (50). Thus, Ace2p is an activator of glycolysis but blocks respiration. Of interest regarding pathogenesis, the *ace1*Δ null mutant was fully capable of germinating within murine macrophages and escaping thereafter, so there was no apparent defect in filamentation. However, under low-O_2 conditions, Ace2p is required for filamentation. The authors also show that filamentation is reduced in the presence of antimycin A, a complex III inhibitor of the CRC chain. Thus, filament formation is hypothesized to occur in wild-type cells by reducing respiration through antimycin A or low oxygen.

Other regulators of glycolysis include Tye7p and Gal4p (3). A double mutant (*tye7*/*gal4*) had a severe growth defect on fermentable carbon sources (glucose, fructose, and mannose) when cells were grown under oxygen-limiting conditions or when respiration was inhibited with antimycin A. The double mutant had a generation time of 210 min in a glucose medium, compared to 139 min for wild-type cells. A reduced growth defect also occurred in the single *tye7*Δ null in the presence of fructose in medium containing antimycin A. Both Tye7 and Gal4p bind to carbohydrate metabolic promoter targets, including those of alcoholic and glucose catabolic, glycolysis, and pyruvate metabolic processes. Tye7p (a basic helix-loop-helix transcription factor) and Gal4p each bind to distinct promoter sequences (motifs) of glycolytic genes. Regulation of target gene promoters was especially reduced in glucose-grown cells of *gal4* and *tye7* null mutants and less so in galactose or glycerol (mitochondria)-grown cells. In spite of the important regulatory activities of both proteins, virulence was only slightly influenced by the loss of either gene; with the double mutant, survival of mice was about 40 to 50% of that obtained with control strains. The remarkable adaptation feature of this pathogen is that it can lose its major glycolytic processes and still retain a significant amount of virulence.

In the case of the Tye7p and Gal4p transcription factors (3), these elements are key regulators of the glycolytic process in *C. albicans*. But, in this regard, each protein has a

distinctive role, Tye7p being a transcriptional global regulator of glycolysis, while Gal4p regulation is carbon source dependent (3).

FROM THE *S. CEREVISIAE* LITERATURE: A LOOK AT HOW MITOCHONDRIA IMPORT PROTEINS

It is possible that mitochondria play a much more important role in the lifestyle of Crabtree-negative organisms, such as *Candida* species, than in Crabtree-positive species, such as *S. cerevisiae*. There are many levels of complexity that could explain this difference among species. In *C. albicans*, transcriptional regulation of glycolysis is at least partially understood, and comparisons have been made with *S. cerevisiae* (3). Glycolysis circuitry is indeed different in these two organisms, but our understanding of the regulation of mitochondrial respiration in *C. albicans* is practically nonexistent. No attempt is made below to present an exhaustive treatise of the total mitochondrial proteome. Instead, we emphasize circuits, beginning with how proteins are translocated and sorted to the various compartments of the *S. cerevisiae* mitochondria.

The mitochondrion is composed of a double membrane, consisting of an OM and an IM. Between the OM and IM is the IMS, and the IM is below the matrix and, importantly, the site of mitochondrial protein synthesis (Fig. 2). Of the ~1,000 mitochondrial proteins in yeast, about 1% are synthesized within the mitochondrial matrix, the rest being imported from the cytoplasm (41). Imported proteins are referred to as N-terminal presequence precursor proteins. The presequence facilitates the binding of proteins to the receptors of the translocases of the outer mitochondrial membrane, or TOM complex (Fig. 2). Imported proteins are distributed to each of the four compartments (OM, IM, IMS, and matrix) by five pathways. Further distinctions are based upon two types of precursor proteins (21). Most IM and matrix proteins have an amino-terminal presequence containing a mitochondrial targeting sequence. Upon import, that sequence is cleaved. The second group of IM, IMS, and OM proteins mostly lack this presequence. However, the targeting and sorting signals fall into at least three categories: the presequences (mentioned above), noncleavable signals of hydrophobic proteins, and internal signals for the IMS site. Sorting of proteins is determined by TOM and TIM (translocase of inner membrane) receptor proteins that

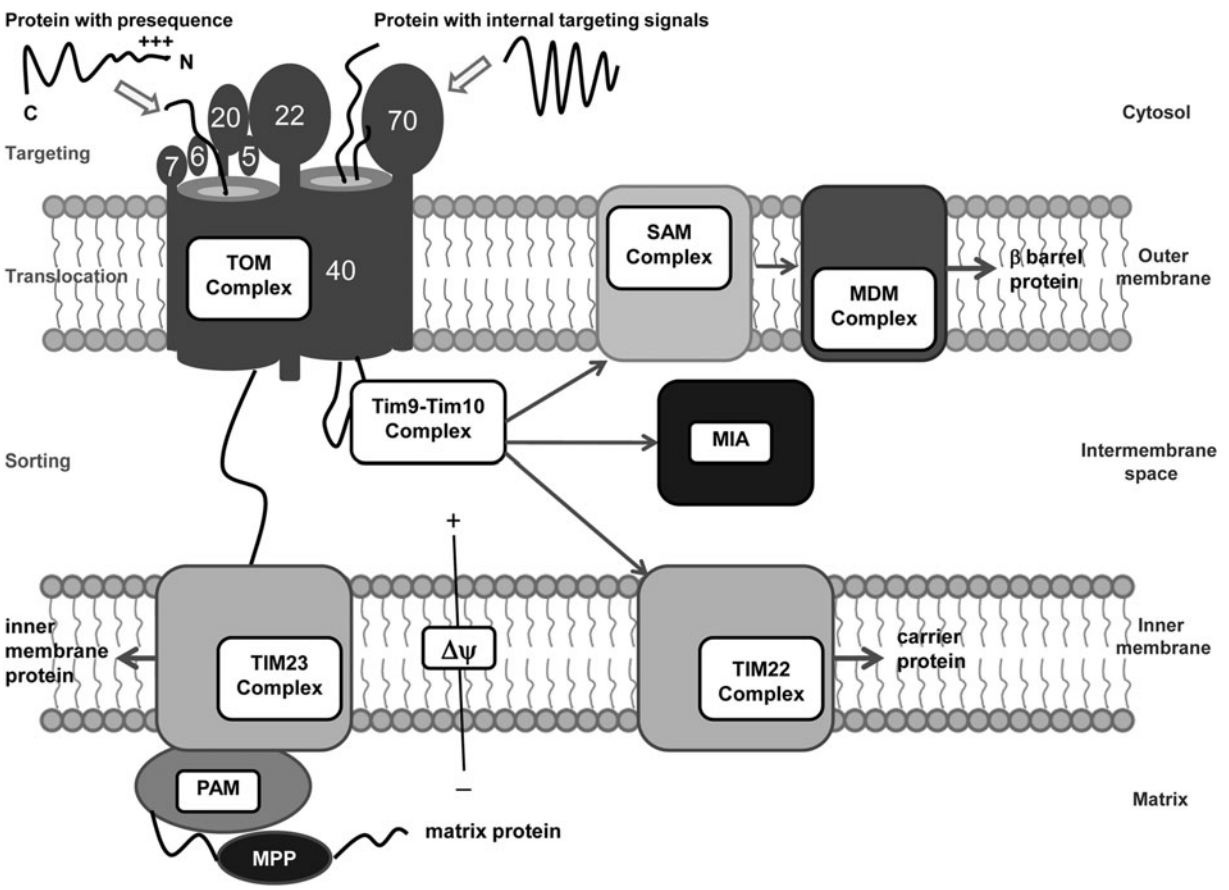

FIGURE 2 Pathways of mitochondrial protein import and distribution to the mitochondria. Translocases are of two types, TOM (of the OM) and TIM (of the IM). SAM, sorting and assembling machinery; MDM, mitochondrial distribution and morphology; PAM, presequence translocase-associated motors; MIA, mitochondrial IMS assembly; MPP, mitochondrial processing peptidase. See reference 13. 10.1128/9781555817176.ch21f2

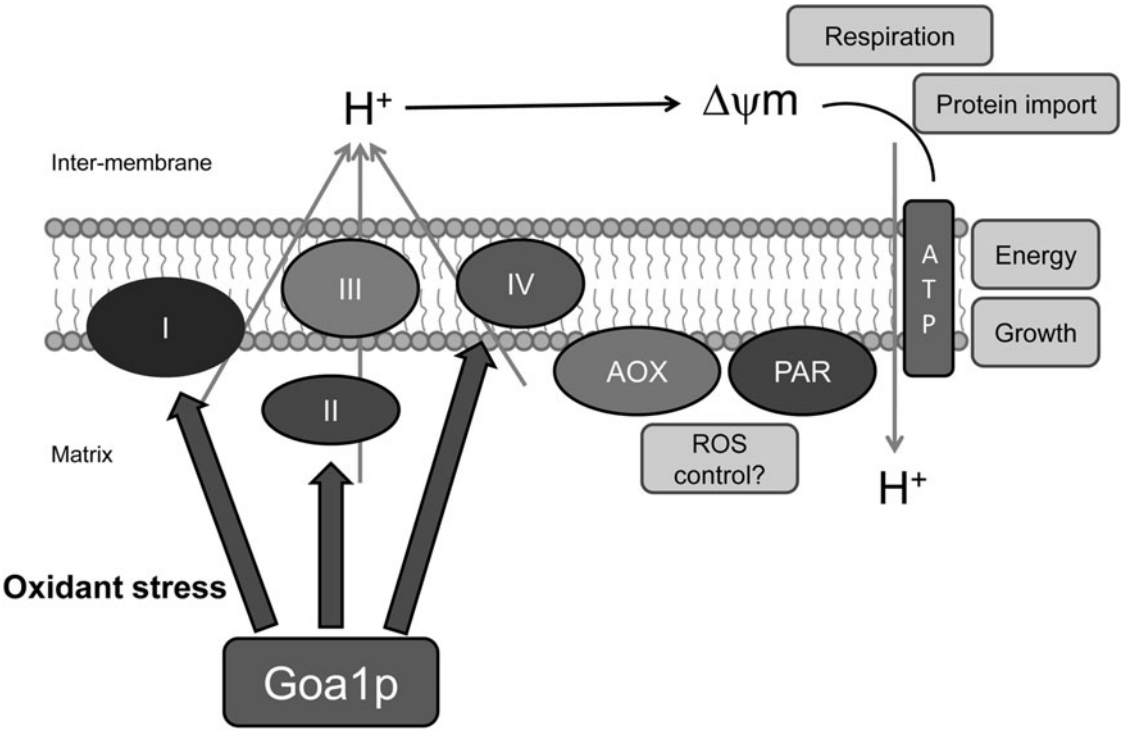

FIGURE 3 Possible roles of Goa1p in mitochondrial function. CRC complexes I to IV as well as AOX and PAR are represented as circles. Since strain GOA31 (*goa1*/*goa1*) is defective in building a membrane potential and ATP formation, we hypothesize that the lesion could be associated with several dysfunctions, including inhibition of complex I, III, or IV, that may lead to the phenotypic traits seen in this mutant. Mitochondrial functions that are affected in the mutant are shown (see also reference 4). 10.1128/9781555817176.ch21f3

act in concert to deliver proteins to their ultimate destination. In Fig. 2, the TOM is pictured as a complex of many shuttle proteins, of which TOM40 appears to be the central component, an oligomer that forms two or three channels per TOM complex (13). Accessory proteins TOM20, TOM22, and TOM70 each play roles in protein sorting to the TOM40 complex. Presequence proteins are recognized on the IMS side of the OM by the TIM23 complex. This, in concert with a variety of other proteins, including MMC/PAM motor proteins, guides presequence proteins through narrow import channels by promoting their unfolding. TIM23 sorts proteins to the mitochondrial IM and matrix, while the TIM22 complex does the same but to the IM only. Of importance to energetic functions, the IM is the site of mitochondrial membrane potential ($\Delta\psi M$) generation through the CRC respiratory pathway complexes (Fig. 1). As expected, TOM40, -70, -22, and -20 and TIM23 and -22 are annotated in both the *C. albicans* (http://www.candidagenome.org/) and *C. dubliniensis* (http://www.sanger.ac.uk/sequencing/Candida/dubliniensis/) databases.

The point of the discussion above is that processes such as import and sorting of mitochondrial proteins are well characterized in *S. cerevisiae* and most likely highly conserved among *Candida* species. There are, however, two points that likely reflect differences among the model yeast and *Candida* species that may reflect the respiratory metabolic regulation. First, the HOG1 mitogen-activated protein kinase (MAPK) pathway has been associated with the control of respiratory metabolism in *C. albicans* (1). No such function has been assigned to the control of respiratory function in the *S. cerevisiae* HOG1 MAPK pathway. The *hog1*Δ and *cap1*Δ null mutants were shown to be sensitive to sodium azide (complex IV), KCN (complex IV and PAR), and oligomycin (ATP synthase) (1) of the CRC respiratory pathway, but the same mutant was insensitive to SHAM, an inhibitor of the AOX pathway. The *hog1*Δ null displayed a higher respiration rate (2.5-fold), an altered membrane potential, a normal level of oxidative phosphorylation, and an increased level of ROS.

The second point is the recent characterization of *GOA1* (growth and oxidant adaptation) from *C. albicans* (4). From null mutants, the functions of Goa1p were discerned as required for optimum growth, oxidant adaptation, yeast-to-hypha formation, survival in neutrophils, and virulence in a murine model of invasive candidiasis. *GOA1* is found in the CUG clade of the Saccharomycotina, which includes five pathogenic *Candida* species (*C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, and *C. lusitaniae* in addition to *C. albicans*) and the nonpathogen *Debaryomyces hansenii* (4, 22). These species use the CUG codon to encode a leucine rather than serine amino acid. *C. glabrata* and *S. cerevisiae*, among other organisms, are not included in this subclade. An association of Goa1p with a mitochondrial function(s) was hypothesized based upon five observations, including that (i) the N terminus of the protein contains a mitochondrial localization domain, and a strain with a green fluorescent protein reporter was shown to shuttle to the mitochondria during oxidant stress; (ii) the null mutant is unable to grow on

media containing nonfermentable carbon sources (glycerol, ethanol, and lactate); (iii) the null mutant fails to generate a membrane potential in the presence of succinate, a substrate of the TCA cycle; (iv) the lack of a substantial membrane potential reduces ATP formation; and (v) oxygen consumption decreases by ~70%. Since *S. cerevisiae* lacks a *GOA1* orthologue, it must mean that regulation of these functions is different in *C. albicans*. In Fig. 3, a model of Goa1p regulation of mitrochondrial activity is shown. Since the *goa1*Δ null lacks membrane potential and produces much less ATP, we suggest that either Goa1p is critical to the integrity of IM proteins, where membrane potential is established, or CRC complexes that provide protons for membrane potential are impaired. In either scenario, the regulation of these activities is different from that in *S. cerevisiae*, since it does not have *GOA1*.

SUMMARY

There is ample proof that mitochondrial functions as well as regulation of these functions vary among pathogenic and model fungi, and that mammalian mitochondria have less complexity with regard to respiratory pathways. This suggests differences that could translate into the development of targets for new drug discovery. Mitochondria of *Candida* species contribute to the growth properties (including morphogenesis), survival of the organism in the presence of oxidant stress, and virulence, and they reduce ROS, maintain survival in the face of carbon scarcity, and modulate the activity of antifungal drugs, depending upon the drug, to make *Candida* species either more sensitive or more resistant. Intriguingly, cancer cells demonstrate a "Warburg effect," wherein enhanced glucose uptake and utilization occur while mitochondria in tumor cells are silenced. Targeting mitochondria to reverse this state may be useful in designing therapies (20, 25). In contrast, studies in animal models link attenuation of mitochondrial respiration in the prevention of pathology due to ischemia-reperfusion injury in myocardial infarction and stroke (26). The point is that mitochondria provide necessary services to cells but can also cause pathology. For these reasons, mitochondria are not to be ignored as we explore the fungal pathogen genomes for ways in which these organism cause diseases and search for new targets and drugs to discover.

REFERENCES

1. **Alonso-Monge, R., S. Carvaihlo, C. Nombela, E. Rial, and J. Pla.** 2009. The Hog1 MAP kinase controls respiratory metabolism in the fungal pathogen *Candida albicans*. *Microbiology* **155:**413–423.
2. **Aoki, S., S. Ito-Kuwa, Y. Nakamura, and T. Masuhara.** 1990. Comparative pathogenicity of a wild type strain and a respiratory mutant of *Candida albicans* in mice. *Zentralbl. Bakteriol.* **273:**332–343.
3. **Askew, C., A. Sellam, E. Epp, H. Hogues, A. Mullick, A. Nantel, and M. Whiteway.** 2009. Transcriptional regulation of carbohydrate metabolism in the human pathogen *Candida albicans*. *PLoS Pathog.* **5:**e1000612.
4. **Bambach, A., M. Fernandes, A. Ghosh, M. Kruppa, D. Alex, D. Li, O. Agrellos, W. Fonzi, R. Rolfes, and R. Calderone.** 2009. Goa1p of *Candida albicans* localizes to the mitochondria during stress and is required for mitochondrial function and virulence. *Eukaryot. Cell* **8:** 1706–1720.
5. **Barelle, C. J., C. L.Priest, D. M. Mccallum, N. A. Gow, F. C. Odds, and A. J. Brown.** 2006. Niche-specific regulation of central metabolic pathways in a fungal pathogen. *Cell. Microbiol.* **8:**961–971.
6. **Batova, M., V. Klobucnikova, Z. Oblasova, J. Gregan, P. Zahradnik, I. Hapala, J. Subik, and C. Schuller.** 2010. Chemogenomic and transcriptome analysis identifies mode of action of the chemosensitizing agent CTBT (7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine). *BMC Genomics* **4:**153–169.
7. **Biggs, D., H. Nakamura, E. Kearney, E. Rocca, and T. Singer.** 1970. Studies on the respiratory chain-linked nicotinamide adenine dinucleotide dehydrogenase. XVI. Characteristics for the membrane-bound dehydrogenase in *Candida utilis* and *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **137:**12–29.
8. **Bouchara, J., R. Zouhair, S. Le Boudouil, G. Renier, R. Filmon, D. Chabasse, J. Hallet, and A. Defontaine.** 2000. *In vivo* selection of an azole-resistant petite mutant of *Candida glabrata*. *J. Med. Microbiol.* **49:**977–984.
9. **Camougrand, N., J. Velours, M. Denis, and M. Guerin.** 1993. Isolation, characterization and function of the 2 cytochrome Cs from *Candida parapsilosis*. *Biochim. Biophys. Acta* **1143:**135–141.
10. **Camougrand, N., S. Zniber, and M. Guerin.** 1991. The antimycin-A-insensitive respiratory pathway of *Candida parapsilosis*: evidence for a second quinine involved specificity in its function. *Biochim. Biophys. Acta* **1057:**124–130.
11. **Cavalheiro, R., F. Borecky, V. Faustinoni, A. Schreiber, and A. Vercesi.** 2004. Respiration, oxidative phosphorylation and uncoupling protein in *Candida albicans*. *Braz. J. Med. Biol. Res.* **37:**1455–1461.
12. **Chabrier-Rosello, Y., T. Foster, S. Mitra, and C. Haidaris.** 2008. Respiratory deficiency enhances the sensitivity of the pathogenic fungus *Candida* to photodynamic treatment. *Photochem. Photobiol.* **84:**1141–1148.
13. **Chacinska, A., C. Koehler, D. Milenkovic, T. Lithgrow, and N. Pfanner.** 2009. Importing mitochondrial proteins: machineries and mechanisms. *Cell* **13:**628–644.
14. **Chamilos, G., R. Lewis, and D. Kontoyiannis.** 2006. Inhibition of *Candida parapsilosis* mitochondrial respiratory pathways enhances susceptibility to caspofungin. *Antimicrob. Agents Chemother.* **50:**744–747.
15. **Chattaway, F. W., R. Bishop, M. R. Holmes, F. C. Odds, and A. J. Barlow.** 1973. Enzyme activities associated with carbohydrate synthesis and breakdown in the yeast and mycelial forms of *Candida albicans*. *J. Gen. Microbiol.* **75:**97–109.
16. **Cheng, S., C. Clancy, K. Nguyen, W. Clapp, and M. Nguyen.** 2007. A petite *Candida albicans* mutant strain with uncoupled oxidative phosphorylation overexpresses MDR1 and has diminished susceptibility to fluconazole and voriconazole. *Antimicrob. Agents Chemother.* **51:** 1855–1858.
17. **Cheng, S. J., C. Clancy, Z. Zhang, B. Hao, W. Wang, K. A. Iczkowski, M. A. Pfaller, and M. H. Nguyen.** 2007. Uncoupling of oxidative phosphorylation enables *Candida albicans* to resist phagocytosis and persist in tissues. *Cell. Microbiol.* **9:**492–501.
18. **Dai, B., Y. Cao, S. Huang, Y. Xu, P. Gao, Y. Wang, and J. Jiang.** 2009. Baicalen induces programmed cell death in *Candida albicans*. *J. Microbiol. Biotechnol.* **19:**803–809.
19. **De Nollin, S., F. Thone, and M. Borgers.** 1975. Enzyme cytochemistry of *Candida albicans*. *J. Histochem. Cytochem.* **23:**758–765.
20. **Derdak, Z., N. Mark, G. Beldi, S. Robson, J. Wands, and G. Baffy.** 2008. The mitochondrial uncoupling protein-2 promotes chemoresistance in cancer cells. *Cancer Res.* **68:**2813–2819.
21. **Endo, T., and K. Yamano.** 2009. Multiple pathways for mitochondrial traffic. *Biol. Chem.* **390:**723–730.

22. **Fitzpatrick, D., M. Logue, J. Stajich, and G. Butler.** 2006. A fungal phylogeny based upon 42 complete genomes derived from supertree and combined gene analysis. *BMC Evol. Biol.* **6:**99–114.
23. **Fradin, G., P. DeGroot, D. MacCallum, M. Schaller, F. Klis, F. C. Odds, and B. Hube.** 2005. Granulocytes govern the transcriptional response, morphology, and proliferation of *Candida albicans* in human blood. *Mol. Microbiol.* **56:**397–415.
24. **Fradin, G., M. Kretschmar, T. Nichterlein, C. Gaillardin, C. d'Enfert, and B. Hube.** 2003. Stage-specific gene expression of *Candida albicans* in human blood. *Mol. Microbiol.* **47:**1523–1543.
25. **Gogvadze, V., S. Orrenius, and B. Zhivotovsky.** 2009. Mitochondria as targets for cancer chemotherapy. *Semin. Cancer Biol.* **19:**57–66.
26. **Gohil, V., S. Sheth, R. Nilsson, A. Wojtovich, J. Lee, F. Perocchi, W. Chen, C. Clish, C. Ayata, P. Brookes, and V. Mootha.** 2010. Nutrient-sensitized screening for drugs that shift energy metabolism from mitochondria respiration to glycolysis. *Nat. Biotechnol.* doi:10.1038/nbt.1606.
27. **Guerin, M. G., and N. M. Camougrand.** 1994. Partitioning of electron flux between the respiratory chains of the yeast *Candida parapsilosis*: parallel working of two chains. *Biochim. Biophys. Acta* **1184:**111–117.
28. **Gyurko, C., U. Lendenmann, R. Troxler, and F. Oppenheim.** 2000. *Candida albicans* mutants deficient in respiration are resistant to the small cationic salivary antimicrobial peptide histatin 5. *Antimicrob. Agents Chemother.* **44:**348–354.
29. **Hamaide-Deplus, M., and E. Nyns.** 1974. Cyanide-insensitive respiration of *Candida lipolytica*. *Antonie van Leeuwenhoek* **40:**79–91.
30. **Helmerhorst, E., M. Stan, M. Murphy, F. Sherman, and F. Oppenheim.** 2005 The concomitant expression and availability of conventional and alternative, cyanide-insensitive, respiratory pathways in *Candida albicans*. *Mitochondrion* **5:**200–211.
31. **Helmerhorst, E., M. Murphy, R. Troxler, and F. Oppenheim.** 2002. Characterization of the mitochondrial respiratory pathways in *Candida albicans*. *Biochim. Biophys. Acta* **1556:**73–80.
32. **Helmerhorst, E., P. Breeuwer, W. van't Hof, E. Walgreen-Weterings, L. Oomen, E. Veerman, A. Amerongen, and T. Abee.** 1999. The cellular target for histatin 5 of *Candida albicans* is the energized mitochondria. *J. Biol. Chem.* **274:**7286–7291.
33. **Huh, W.-K., and S. Kang.** 2001. Characterization of a gene family encoding alternative oxidase from *Candida albicans*. *Biochem. J.* **356:**595–604.
34. **Huh, W.-K., and S. Kang.** 1999. Molecular cloning and functional expression of an alternated oxidase from *Candida albicans*. *J. Bacteriol* **181:**4098–4102.
35. Reference deleted.
36. **Jarmuszkiewicz, W., G. Milani, F. Fortes, A. Schreiber, F. Sluse, and A. Vercesi.** 2000. First evidence and characterization of an uncoupling protein in the fungal kingdom. *FEBS Lett.* **467:**145–149.
37. **Jia, J., Y. Wang, Y. Cao, P. Gao, X. Jia, Z. Ma, Y. Xu, B. Dai, and Y. Jiang.** 2007. CalPF7817 is involved in the regulation of redox homeostasis in *Candida albicans*. *Biochem. Biophys. Res. Commun.* **359:**163–167.
38. **Joseph-Horne, T., D. Hollomon, and P. Wood.** 2001. Fungal respiration: a fusion of standard and alternative components. *Biochim. Biphys. Acta* **1504:**179–195.
39. **Kaur, R., I. Castaño, and B. P. Cormack.** 2004. Functional genomic analysis of fluconazole susceptibility in the pathogenic yeast *Candida glabrata*: roles of calcium signaling and mitochondria. *Antimicrob. Agents Chemother.* **48:**1600–1613.
40. **Konopka, K., B. Dorocka-Bobkowska, S. Gebremedhin, and N. Düzgüneş.** 2010. Susceptibility of *Candida* biofilms to histatin 5 and fluconazole. *Antonie van Leeuwenhoek* doi:10.1008/s10482-010-9417-5.
41. **Kot, E., J. Rolewic, V. Olson, and D. McClary.** 1975. Growth, respiration and cytology of acetate-negative mutants of *Candida albicans*. *Antonie van Leewenhoek* **41:**229–248.
42. **Kutik, S., B. Guiard, B. Meyer, N. Wiedemann, and N. Pfanner.** 2007. Cooperation of translocase complexes in mitochondrial protein import. *J. Cell Biol.* **179:**585–591.
43. **Land, G., W. McDonald, R. Stjernholm, and L. Friedman.** 1975. Factors affecting filamentation in *Candida albicans*. Relationship of the uptake and distribution of proline to morphogenesis in *Candida albicans*. *Infect. Immun.* **11:**1014–1023.
44. **Land, G., W. McDonald, R. Stjernholm, and L. Friedman.** 1974. Factors affecting filamentation in *Candida albicans*: changes in respiratory activity of *Candida albicans* during filamentation. *Infect. Immun.* **12:**119–127.
45. **Lorenz, M., J. Bender, and G. Fink.** 2004. Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot. Cell* **3:**1076–1087.
46. **Martchenko, M., A. Alarco, D. Harcus, and M. Whiteway.** 2004. Superoxide dismutases in *Candida albicans*: transcriptional regulation and functional characterization of the hyphal-induced *SOD5* gene. *Mol. Biol. Cell* **15:** 456–467.
47. **McDonough, J., V. Bhattacherjee, T. Sadlon, and M. Hostetter.** 2002. Involvement of *Candida albicans* NADH dehydrogenase complex I in filamentation. *Fungal Genet. Biol.* **36:**117–127.
48. **Milani, G., W. Jarmuskiewicz, C. Sluse-Goffart, A. Schreiber, A. Vercesi, and F. Sluse.** 2001. Respiratory chain network in mitochondria of *Candida parapsilosis*: ADP/O appraisal of the multiple electron networks. *FEBS Lett.* **508:**231–235.
49. **Mulhern, S., M. Logue, and G. Butler.** 2006. *Candida albicans* transcription factor Ace2 regulates metabolism and is required for filamentation in hypoxic conditions. *Eukaryot. Cell* **5:**2001– 2013.
50. **Nyns, E., and M. Hamaide.** 1972. Cyanide-insensitive respiration of *Candida lipolytica*. *Arch. Int. Physiol. Bochim.* **80:**978–980.
51. **Palou, C., M. Pico, M. Bonet, and P. Olover.** 1998. The uncoupling protein, thermogenin. *Int. J. Biochem. Cell Biol.* **30:**7–11.
52. **Quail, J., C. McQuillan, C. Munro, A. Pain, R. Poulter, H. Rajandream, H. Renauld, M. Spiering, A. Tivey, N. Gow, B. Barell, D. Sullivan, and M. Berriman.** 2009. Comparative genomics of the fungal pathogens *Candida dubliniensis* and *Candida albicans*. *Genome Res.* **19:** 2231–2234.
53. **Ramirez, M., and M. Lorenz.** 2007. Mutations in alternative carbon utilization pathways in *Candida albicans* attenuate virulence and confer pleiotropic phenotypes. *Eukaryot. Cell* **6:**280–290.
54. **Rikhvanov, E. G., N. N. Varakina, T. M. Rusaleva, E. I. Rachenko, D. A. Knorre, and V. K. Voinikov.** 2005. Do mitochondria regulate heat shock response in *Saccharomyces cerevisiae*? *Curr. Genet.* **48:**44–59.
55. **Ruy, F., A. Vercesi, and A. Kowaltowski.** 2006. Inhibition of specific electron transport pathways leads to oxidative stress and decreased *Candida albicans* proliferation. *J. Bioenerg. Biomembr.* **38:**129–135.

56. **Sanglard, D., F. Ischer, and J. Bille.** 2001. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. *Antimicrob. Agents Chemother.* **45:**1174–1183.
57. **Santos, R., N. Buisson, S. Knight, A. Dancis, A. Camado, and E. Lesuisse.** 2004. *Candida albicans* lacking the frataxin homologue: a relevant yeast model for studying the role of frataxin. *Mol. Microbiol.* **54:**507–519.
58. **Shepard, M., and P. Sullivan.** 1976. The production and growth characteristics of yeast and mycelial forms of *Candida albicans* in continuous culture. *J. Gen. Microbiol.* **93:**361–370.
59. **Shepard, M., C. Chin, and P. Sullivan.** 1976. The alternative respiratory pathway of *Candida albicans*. *Arch. Microbiol.* **116:**61–71.
60. **Shigematsu, M., J. Uno, and T. Arai.** 1982. The effect of ketoconazole on isolated mitochondria from *Candida albicans*. *Antimicrob. Agents Chemother.* **21:**919–924.
61. **Shimiada, H., K. Kondo, P. Fraser, Y. Miura, T. Saito, and N. Misawa.** 1998. Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. *Appl. Environ. Microbiol.* **64:** 2676–2680.
62. **Swoboda, R., G. Bertram, S. Delbruck, J. Ernst, N. Gow, G. Gooday, and A. Brown.** 1994. Fluctuations in glycolytic mRNA levels during morphogenesis in *Candida albicans* reflect underlying changes in growth and are not a response to cellular dimorphism. *Mol. Microbiol.* **13:** 663–672.
63. **Vellucci, V., S. Gygax, and M. Hostetter.** 2007 Involvement of *Candida albicans* pyruvate dehydrogenase complex protein X (Pdx1) in filamentation. *Fungal Genet. Biol.* **44:**979–990.
64. **Vercesi, A., J. Borecky, G. Maia, P. Arrunda, I. Cuccovia, and H. Chaimovich.** 2006. Plant uncoupling mitochondrial proteins. *Annu. Rev. Plant Biol.* **57:**383–404.
65. **Ward, J., and W. Nickerson.** 1957. Respiratory metabolism of normal and divisionless strains of *Candida albicans*. *J. Gen. Physiol.* **41:**703–725.
66. **Weiss, E., C. Kurischko, C. Zhang, K. Shokat, D. Drubin, and F. Luca.** 2002. The *Saccharomyces cerevisiae* Mob-2pCbk1p kinase complex promotes polarized growth and acts with the mitotic exit network to facilitate daughter-cell-specific localization of Ace2p transcription factor. *J. Cell Biol.* **158:**885–900.
67. **Wu, X., A. Cheng, L. Sun, S. Sun, and H. Lou.** 2009. Plagiochin E, an antifungal bis(bibenzyl), exerts its antifungal activity through mitochondrial dysfunction-induced reactive oxygen species accumulation in *Candida albicans*. *Biochim. Biophys. Acta* **1790:**770–777.
68. **Wu, Z., W. Chang, A. Cheng, L. Sun, and H. Lou.** 2010. Plagiochin E, an antifungal active macrocyclic bis(bibenzyl), induced apoptosis in *Candida albicans* through a metacaspase-dependent apoptotic pathway. *Biochim. Biophys. Acta* **1800:** 439–447.
69. **Xie, C., Z. Wu, N. Liu, M. Ji, and H. Lou.** 2010. Antifungal macrocyclic bis(bibenzyls) from the Chinese liverwort *Ptagiochasm intermedlum* [sic] *L. Nat. Prod. Res.* **23:**1–6.
70. **Yan, L., M. Li, Y. Cao, P. Cao, Y. Wang, and Y. Jiang.** 2009. The alternative oxidase of *Candida albicans* causes reduced fluconazole susceptibility. *J. Antimicrob. Chemother.* **64:**764–773.

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ANTIFUNGAL DRUGS, DRUG RESISTANCE, AND DISCOVERY

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy
©2012 ASM Press, Washington, DC
10.1128/9781555817176.ch22

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Antifungals: Drug Class, Mechanisms of Action, Pharmacokinetics/Pharmacodynamics, Drug-Drug Interactions, Toxicity, and Clinical Use

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HISTORICAL OVERVIEW

The incidence of invasive fungal infections has continued to rise in parallel with an increase in the susceptible immunocompromised host population (152). Antifungal therapeutic outcomes have been historically suboptimal, in part, due to a relatively small number of safe and effective antifungal drugs. A decade ago, there were only three drug classes and seven approved antifungal agents for treatment of systemic fungal infections. The development of amphotericin B in the 1950s was a major advance. Until recently, formulations of this potent, broad-spectrum antifungal were the standard therapy for the treatment for most life-threatening invasive fungal infections. However, dose-limiting toxicities that include serious infusion reactions, electrolyte abnormalities, and renal failure (in up to 50% of patients) have limited the utility of this agent (33).

The 1990s saw both the refinement of the amphotericin B formulations and the introduction of the triazole drug class (Table 1). Because of the reduced nephrotoxicity of the lipid-based amphotericin B formulations in comparison with that of the parent formulation (10- to 20-fold less), these compounds allowed for the infusion of higher dosages of amphotericin B. In general, the triazoles offered a broad antifungal spectrum, the potential for oral administration, and reduced toxicity compared to the amphotericin B formulations. For treatment of many systemic mycoses, the triazoles remain effective and safe alternatives to the older antifungal drugs, amphotericin B and flucytosine, a fluorinated pyrimidine.

Although the triazole drugs fluconazole and itraconazole have excellent activity against *Candida* species and the endemic fungi, these agents have limited utility in the treatment of mycoses due to the emerging filamentous fungal pathogens. The expanded-spectrum azole drugs were designed to have extended activities against mold pathogens and emerging *Candida* spp. Voriconazole, approved in 2002, was the first of these to become commercially available, and posaconazole was licensed in 2006 (122, 162). In addition, a new class of drugs, the echinocandins, has been developed. These agents disrupt fungal cell wall β-1,3 glucan and demonstrate activity against some pathogenic molds and *Candida* spp., including *Candida glabrata*. Three drugs of the echinocandin class, caspofungin, micafungin, and anidulafungin, have been licensed for clinical use. As drug-resistant fungi emerge, novel agents with enhanced activities will be of interest.

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PHARMACOLOGICAL AND CLINICAL CHARACTERISTICS

There are many important characteristics of antifungal drugs to consider in treatment of invasive fungal infection. Among these traits, spectrum of activity, pharmacokinetics, pharmacodynamics, potential drug-drug interactions, and toxicities are the most critical. The spectra of activity vary among the antifungal classes and within the individual classes. Organisms may be intrinsically resistant to an antifungal, or resistance may develop upon exposure. The term pharmacokinetics describes the process by which a drug is absorbed, distributed, metabolized, and eliminated in the host, and the term pharmacodynamics correlates drug pharmacokinetics, in vitro susceptibility, and treatment outcome. The clinical importance of these two characteristics is discussed below. Safe drug administration requires knowledge of likely toxicities and potential interactions with other drugs due to altered metabolism or increased risk of toxicity. These antifungal drug characteristics are considered with clinical data for the development of recommendations and the approval of antifungals for treatment of specific fungal infections. This chapter focuses on these antifungal traits for available systemic agents for treatment of *Candida* infection.

TABLE 1 History of antifungal therapy

Antifungal	Class	Year(s)	Major advantage(s)	Major drawback(s)
Amphotericin B deoxycholate	Polyene	1958	Potent, broad-spectrum activity	Renal toxicity Serious infusion reaction
Flucytosine	Pyrimidine analog	1973	Activity against *Candida* and *Cryptococcus*	Rapid emergence of resistance as monotherapy Toxicity
Fluconazole	Azole	1990	Good oral bioavailability Well tolerated Central nervous system penetration Activity against yeast	Limited activity against filamentous fungi CYP450 interactions
Itraconazole	Azole	1992	Variable oral bioavailability Activity against filamentous and endemic fungi	Variable oral bioavailability CYP450 interactions
Lipid formulations of amphotericin B	Polyene	1995–1997	Less toxicity than deoxycholate formulation	High dose required for efficacy High cost
Voriconazole	Azole	2002	Potent *Candida* activity Parenteral and oral formations available	Variable metabolism and oral bioavailability
Posaconazole	Azole	2006	Activity against zygomycetes	Oral formation only Difficult to achieve therapeutic levels rapidly due to saturable absorption
Caspofungin Micafungin Anidulafungin	Echinocandin	2001–2006	Well tolerated Few drug-drug interactions Good activity against *C. glabrata*	Variable activity against *C. parapsilosis* Parenteral formulations only

PHARMACOKINETICS AND PHARMACODYNAMICS

The role of pharmacokinetics and pharmacodynamics has gained increasing recognition as critical for selection and dosing of antimicrobial therapeutics, including antifungal agents. Knowledge of pharmacokinetic/pharmacodynamic indices associated with efficacy can be helpful for clinicians to predict therapeutic success, guide optimal dosing levels and intervals, aid in susceptibility breakpoint development, direct therapeutic drug monitoring, and limit potential adverse outcomes, including toxicity and the drug resistance.

Pharmacodynamics examines the relationship between pharmacokinetics and outcome. An added dimension of antimicrobial pharmacodynamics is consideration of the drug exposure relative to a measure of in vitro potency, or the MIC. Three pharmacodynamic indices have been used to describe these relationships, including the peak concentration in relation to the MIC (C_{max}/MIC), the area under the concentration curve in relation to the MIC (24-h area under the concentration curve, AUC/MIC), and the time that drug concentrations exceed the MIC, expressed as a percentage of the dosing interval (%*T*>MIC). Knowledge of which of the three pharmacodynamic indices best describes antifungal activity provides the basis for determining the frequency with which a drug is most efficaciously administered. For example, if the C_{max}/MIC relationship strongly correlates with activity of drug A, the optimal dosing schedule would provide large infrequent doses. Conversely, if the %*T*>MIC better describes drug activity, a dosing strategy may include smaller, more frequent, or even continuous drug administration to prolong the period that drug levels exceed the MIC.

Several experimental designs are used to define the pharmacodynamic index linked to drug efficacy. In the first, the impact of escalating antifungal drug concentrations on fungal viability is observed over time. For some drugs, increasing drug concentrations above the MIC enhances the rate and extent of organism death. When higher concentrations enhance killing, the pharmacodynamic pattern of activity is termed concentration-dependent killing. The second observation is undertaken long after drug exposure (after the antimicrobial is no longer present or is present at concentrations below the MIC). For some compounds there is a period of prolonged growth suppression following the initial supra-MIC exposure. This period of growth suppression is termed a postantifungal effect (PAFE). Three combinations of these time-kill endpoint characteristics have been described, and each combination is typically associated with one of the pharmacodynamic indices. The C_{max}/MIC is associated with concentration-dependent killing and prolonged PAFEs. The %*T*>MIC is associated with concentration-independent killing and short PAFEs. The AUC/MIC is associated with prolonged PAFEs and either concentration-dependent or -independent killing.

A final experimental design is termed dose fractionation. Traditional dose escalation studies use a single dosing interval. With only a single dosing interval, escalating doses increase the values of all three indices. Dose fractionation studies examine efficacy of various dose levels that are administered by using three or more dosing intervals. In examining treatment results, if the regimens with shorter dosing intervals are more efficacious, the time-dependent index (%*T*>MIC) is the more important index. If the large, infrequently administered dosing regimens are more active, the

peak level in relation to the MIC is most predictive. Finally, if the outcome is similar with each of the dosing intervals, the outcome depends on the total dose or the AUC for the dosing regimen.

As mentioned above, knowledge of the pharmacodynamic characteristics of a compound allows one to better design a dosing interval strategy. This knowledge can also be useful to design studies to answer questions regarding the amount of drug or index magnitude that is associated with treatment efficacy. For example, what pharmacodynamic magnitude of a drug is needed to treat a *Candida* infection? Is this pharmacodynamic magnitude the same as that needed to treat a drug-resistant *Candida* infection? Is the magnitude similar for other fungal species, for different infection sites, and in different animal species? The answers to these questions have been explored and, in general, successfully addressed using a variety of infection models. The results of these studies have demonstrated that the magnitudes of a pharmacodynamic index associated with efficacy are similar for drugs within the same class, provided that free drug (non-protein-bound) levels are considered. The pharmacodynamic evaluation of each antifungal drug class and the clinical implications of these studies are briefly discussed in the following sections.

POLYENES

The only molecule in the polyene drug class that is available for treatment of systemic fungal infections is amphotericin B. In addition to the original deoxycholate formulation of amphotericin B, three lipid formulations have been developed to improve the safety margin of these compounds.

Mechanism of Action

Polyenes are a class of natural products from *Streptomyces nodosus*, a soil actinomycete (Fig. 1) (89). Amphotericin B acts by binding to the ergosterol cell membrane component of fungi and perturbing membrane competency. Pores formed by these complexes disrupt membrane stability and allow the efflux of intracellular material, leading to cell death (25).

Spectrum of Activity

Amphotericin B demonstrates potent activity against most pathogenic yeasts and filamentous fungi (Table 2). Among the antifungal drug classes, amphotericin B has the broadest spectrum of activity and resistance rates are low. Amphotericin B is active against most *Candida* spp. and demonstrates low MICs for *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (Table 3). One major exception is *C. lusitaniae*, for which MICs are consistently higher (73, 190). Amphotericin B also demonstrates activity against most other pathogenic yeasts, including *Cryptococcus* spp. (Table 2) (195). The drug is active against most dimorphic fungi, including *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Sporothrix schenckii*, and *Paracoccidioides* spp. (142, 151, 224). Amphotericin B is also active against most *Aspergillus* spp., with the exception of *A. terreus* (224). The compound has limited activity against *Scedosporium* spp. and *Fusarium* spp. but demonstrates activity against many species in the Zygomycetes group of fungi (6, 73, 153, 224).

Clinically Relevant Pharmacokinetics

Amphotericin B displays poor solubility and oral bioavailability and is available solely as an intravenous (i.v.) formulation. Peak levels are achieved approximately 4 to 6 h after infusion, and drug levels remain elevated for 6 to 8 h (89). The formulations are all highly protein bound (>95%) and have long half-lives, allowing for once-daily dosing (254). Although levels of amphotericin B in the cerebrospinal fluid are nearly undetectable, it is the drug of choice for treatment of cryptococcal meningitis (26, 48). This apparent discrepancy is presumably because the high brain parenchymal drug concentrations are a more accurate predictor of therapeutic response.

Each of the lipid formulations exhibits distinct pharmacokinetics. However, one similarity is the propensity to accumulate in the reticuloendothelial system (121). In addition, the lipid carrier molecules reduce binding to the renal tubular cells compared to the deoxycholate formulation. The latter pharmacokinetic difference is theorized to result in less nephrotoxicity. One of the notable differences among the three available amphotericin B lipid preparations is the increased penetration of liposomal amphotericin B into the central nervous system. Administration of this formulation results in approximately four- to sevenfold-higher brain parenchymal concentrations compared to the administration of the deoxycholate preparation or the other lipid-based preparations. This tissue kinetic difference has been shown to offer a therapeutic advantage in an animal model of *Candida* meningitis (25, 99).

The routes of metabolism and elimination of the amphotericin B formulations are not entirely understood. The deoxycholate preparation is primarily excreted as the parent drug into the feces (43%) and urine (21%) (34). However, only 10% of the liposomal amphotericin B formulations is excreted in the unchanged form, suggesting altered metabolism for the lipid formulations. Although dose adjustments are not required in patients with hepatic or renal failure, use of this class in patients with renal insufficiency is cautioned due to the increased likelihood of additional nephrotoxicity (Table 4).

Pharmacodynamics

The impact of escalating polyene concentrations has been well studied using in vitro polyene time-kill study design (Table 5) (12, 19, 79, 80, 99, 127, 141, 262, 278, 288). These studies have demonstrated marked concentration-dependent killing and maximal antifungal activity at concentrations exceeding the MIC from 2- to 10-fold. Results from these studies have also demonstrated prolonged persistent growth suppression following drug exposure and removal (12). Dose fractionation studies in which the dosing interval is varied widely in treatment of murine invasive candidiasis have demonstrated optimal efficacy with administration of large, infrequent doses. Increasing the dosing interval from every 12 h to every 72 h enhanced the efficacy and decreased the total amount of amphotericin B needed to inhibit organism growth in animals by a factor of 10. These pharmacodynamic data indicate that the C_{max}/MIC index best predicts efficacy (12). In vivo study with amphotericin B and multiple *Candida* species in a neutropenic disseminated candidiasis model demonstrated growth inhibition when the C_{max}/MIC ratio approached values as low as 2 and a maximal reduction in organism burden when this ratio approached a value of 10.

In vivo studies have demonstrated that the lipid formulations of amphotericin B are not as potent in vivo as conventional amphotericin B on a weight (milligram/kilogram) basis. Animal model pharmacodynamic studies suggest that the lipid amphotericin B exposure needed for in vivo efficacy

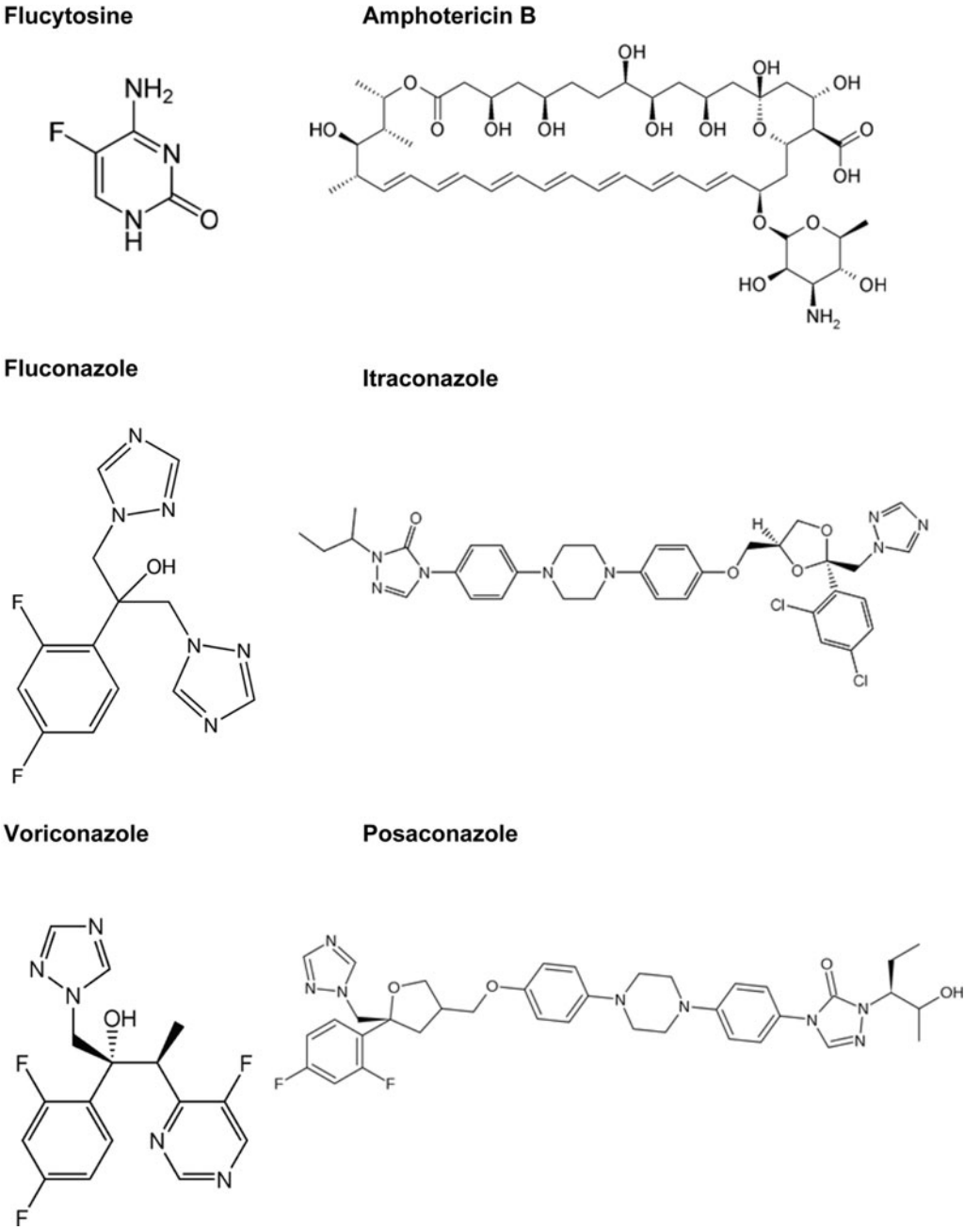

FIGURE 1 Chemical structures of systemically available antifungal agents. 10.1128/9781555817176.ch22f1A

is roughly four to five times higher than that observed for the deoxycholate formulation (19).

Few clinical investigations have sought a connection among amphotericin B kinetics, in vitro susceptibility, and clinical outcome (28, 177). A small pediatric patient study did link patient liposomal amphotericin B pharmacokinetics and organism MIC to patient outcome (113). The results demonstrated a statistically significant correlation between C_{max}/MIC ratio and outcome, with maximal efficacy observed at serum C_{max}/MIC ratios greater than 40, consistent with data from the animal model studies (19).

Toxicities

The adverse effects of amphotericin B often limit its use. The main toxicities include renal damage, electrolyte abnormalities from renal toxicity, hepatotoxicity, and infusion-related reactions (33, 53). Toxicity to the kidneys results from two mechanisms. First, osmotic changes in the tubular system result in constriction of the afferent blood supply via a mechanism termed tubuloglomerular feedback (49). The second mechanism of toxicity is the result of direct tubular cell effects of amphotericin B. The resulting tubular cell damage is felt to be a result of electrolyte disturbances in

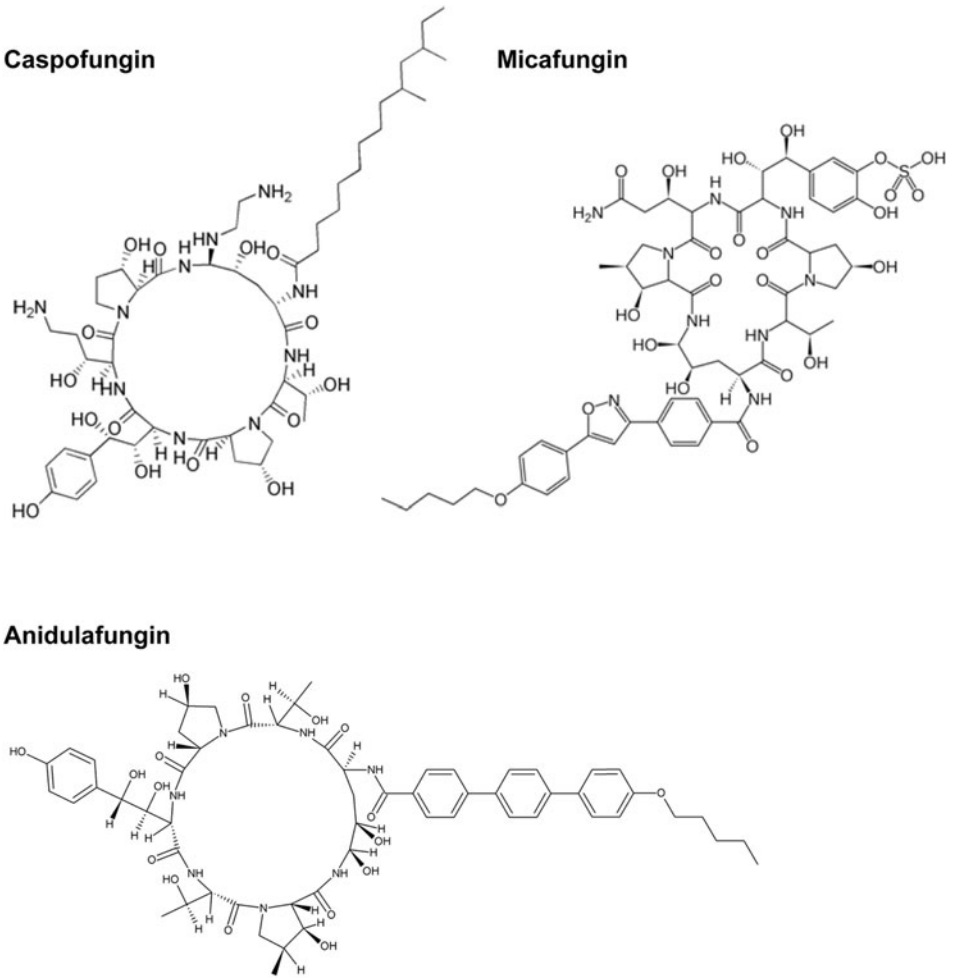

FIGURE 1 *(Continued)* 10.1128/9781555817176.ch22f1B

TABLE 2 Spectra of activity for systemic antifungal agents[a]

Organism	AMB	5FC	FLU	ITR	VOR	POS	CAS	MC	ANI
C. albicans	+	+	+	+	+	+	+	+	+
C. glabrata	+	+	+/–	+/–	+	+	+	+	+
C. parapsilosis	+	+	+	+	+	+	+/–	+/–	+/–
C. tropicalis	+	+	+	+	+	+	+	+	+
C. krusei	+	+/–	–	+/–	+	+	+	+	+
C. lusitaniae	–	+	+	+	+	+	+	+	+
A. fumigatus	+	–	–	+	+	+	+	+	+
C. neoformans	+	+	+	+	+	+	–	–	–
Zygomycetes	+/–	–	–	–	–	+	–	–	–
Fusarium spp.	+/–	–	–	–	+	+	–	–	–
Scedosporium spp.	+/–	–	–	+/–	+	+	–	–	–
H. capsulatum	+	–	+	+	+	+	–	–	–
B. dermatitidis	+	–	+	+	+	+	–	–	–
C. immitis	+	–	+	+	+	+	–	–	–

[a]AMB, amphotericin B; 5FC, flucytosine; FLU, fluconazole; ITR, itraconazole; VOR, voriconazole; POS, posaconazole; CAS, caspofungin; MC, micafungin; ANI, anidulafungin.

TABLE 3 In vitro susceptibilities of *Candida* spp. to various antifungals

Species	MIC_{90} range (total MIC range), in μg/ml, for antifungal agent[a]								
	AMB	5FC	FLU	ITR	VOR	POS	CAS	MC	ANI
C. albicans	0.25–1 (0.06–4)	1 (0.12–>128)	0.25–8 (0.06–>128)	0.03–0.5 (0.01–>8)	0.02–0.5 (0.007–>16)	0.02–0.12 (0.007–>8)	0.06–0.5 (0.007–>8)	0.03 (0.007–0.5)	0.03–0.06 (0.007–1)
C. glabrata	0.5–4 (0.06–16)	0.12 (0.06–8)	8–64 (0.12–>128)	1–4 (0.06–>8)	0.25–2 (0.02–>8)	1–2 (0.007–>8)	0.25–1 (0.007–>8)	0.06 (0.007–2)	0.12 (0.02–4)
C. parapsilosis	0.5–4 (0.25–4)	0.12–0.25 (0.12–0.5)	1–8 (0.12–128)	0.06–0.5 (0.02–2)	0.03–0.12 (0.007–>8)	0.03–0.25 (0.02–1)	2–4 (0.02–>8)	2 (0.02–2)	2 (0.02–4)
C. tropicalis	0.5–2 (0.5–4)	0.5–1 (0.12–16)	2–16 (0.12–>128)	0.06–1 (0.02–>8)	0.06–2 (0.007–>16)	0.06–1 (0.02–>8)	0.5–1 (0.007–>8)	0.06 (0.007–1)	0.12 (0.007–2)
C. dubliniensis	0.12–0.5 (0.06–1)	0.06–0.12 (0.06–0.5)	0.5–16 (0.12–>128)	0.06–0.25 (0.02–0.5)	0.03 (0.007–>8)	0.06 (0.02–>8)	0.5 (0.02–1)	0.03	0.06
C. krusei	0.5–8 (0.06–16)	32 (0.06–>128)	64–>128 (2–>128)	0.25–4 (0.12–4)	0.5–2 (0.06–4)	0.25–1 (0.12–2)	0.25–2 (0.02–4)	0.25 (0.02–0.25)	0.12 (0.02–0.5)
C. lusitaniae	0.5–1 (0.06–16)	8 (0.06–>128)	2–4 (0.12–64)	0.06–0.5 (0.03–0.5)	0.02–0.06 (0.007–2)	0.02–0.12 (0.02–1)	0.25–2 (0.03–2)	2 (0.007–2)	0.25 (0.06–1)
C. guilliermondii	0.06–1 (0.06–32)	0.12–0.5 (0.06–4)	4–16 (0.25–>128)	0.5–1 (0.03–1)	0.12–0.5 (0.007–>8)	0.06–0.5 (0.02–8)	1–>8 (0.03–>8)	0.5 (0.015–>8)	1 (0.06–4)
C. famata			16 (0.25–16)	1 (0.06–2)		1 (0.02–1)	(0.06–>8)		
C. rugosa	4 (0.5–32)	16 (0.06–32)	16 (0.5–32)		0.12 (0.02–0.5)	0.25 (0.02–0.5)	(0.12–0.5)		
C. pelliculosa			8 (2–8)	1–>8	0.25–>8 (0.007–1)	0.5–>8 (0.007–>8)			

[a]Data taken from references 46, 122, 185, 187, 188, 191, 193, 194, 197, and 199. For abbreviations, see Table 2, footnote *a*.

the renal tubular cells due to the presence of membrane pores created by the association of amphotericin B with membrane cholesterol (298). The renal toxicity of amphotericin B increases as the total cumulative dose increases, with risk of irreversible damage reported when the total accumulative dose reaches 3 to 4 g (53). Examination of amphotericin B administration at tertiary care hospitals found a 30% rate of acute renal failure linked to administration of the drug (33). Renal failure in this setting was associated with a high mortality rate (54%). The rate of nephrotoxicity is substantially lower for neonates, and amphotericin B can be safely administered for treatment of neonatal candidiasis (143, 176). The mechanism of this difference in toxicity between neonates and adults has not been extensively studied.

Although the lipid-based formulations are less nephrotoxic, they are not without side effects (25, 180). Like the deoxycholate preparation, the lipid-based formulations are also associated with infusion-related toxicity. While hepatotoxicity has been reported following administration of amphotericin B deoxycholate or the liposomal preparations, this rarely requires drug discontinuation (90).

Drug-Drug Interactions

Amphotericin B is not a major substrate or inhibitor of the hepatic cytochrome P450 (CYP450) enzymes and exhibits few drug-drug interactions (Table 6). The majority of the drug-drug interactions are related to potentiation of the electrolyte disturbances and renal dysfunction typical of amphotericin B. For example, the risk of renal toxicity is increased if amphotericin B is used concomitantly with the organ transplant immunosuppressants cyclosporine and tacrolimus (1).

Clinical Uses

Amphotericin B deoxycholate was one of the first drugs developed for the treatment of invasive fungal infections and has been approved for many applications, including invasive candidiasis, aspergillosis, cryptococcosis, blastomycosis, histoplasmosis, zygomycosis, and sporotrichosis. However, toxicity of the deoxycholate formulation often requires dose reduction, which can lead to therapeutic failure (171). The lipid-based preparations of amphotericin B have reduced toxicity compared to the deoxycholate formulation and have in large part supplanted use of the deoxycholate formulation for the same indications (171). However, the lipid-based formulations are more expensive and not entirely free of side effects.

Amphotericin B has been studied extensively and shown to be effective for the treatment of candidemia and invasive candidiasis (89, 169, 258, 282). The lipid-based amphotericin B formulations are currently recommended as a first-line therapy for treatment of candidemia or suspected invasive candidiasis in neutropenic patients (176). Amphotericin B deoxycholate is considered a second-line or alternative therapy for nonneutropenic patients with suspected invasive candidiasis or candidemia. The original deoxycholate formulation, however, remains the first-line choice for treatment of neonatal disseminated candidiasis due to the enhanced safety profile in this population (143, 176). In addition, these formulations are preferred for treatment of pregnant patients due to gestational toxicity with the triazole drug class. Liposomal amphotericin B is recommended for treatment of candidiasis involving the central nervous system, eyes, or cardiovascular system (45, 156, 165, 176).

This class has many non-*Candida* indications as well. Amphotericin B is used in combination with flucytosine for

TABLE 4 Dosing regimens and clinical indications for frequently used systemic antifungal agents[a]

Agent	Adult dosing		Pediatric dosing	Renal insufficiency	Hepatic insufficiency	Clinical indications
	i.v.	Oral				
Amphotericin B	0.25–1 mg/kg/day	NA	0.7–1 mg/kg/day	Decrease dosing interval to q48h for CrCl of <10	No adjustments	Aspergillosis Mucosal candidiasis Invasive candidiasis Cryptococcosis Coccidioidomycosis Blastomycosis Histoplasmosis Mucormycosis
Flucytosine	NA	25 mg/kg 4 times/day	25 mg/kg 4 times/day	Decrease dosing interval to q12–48h	No adjustments	Cryptococcosis (in combination therapy) Second line for candidiasis
Fluconazole	200–800 mg daily	200–800 mg daily	3–12 mg/kg daily	Decrease dose by half for CrCl of <50	No adjustments	Mucosal candidiasis Invasive candidiasis Cryptococcosis Coccidioidomycosis
Itraconazole	NA	200 mg 1–3 times/day	2.5–5 mg/kg 2 or 3 times/day	Avoid i.v. formulation for CrCl of <30	No adjustments	Mucosal candidiasis Histoplasmosis Blastomycosis Second line for aspergillosis Onychomycosis Paracoccidioidomycosis Coccidioidomycosis
Voriconazole	6-mg/kg load for 2 doses 4 mg/kg q12h	400-mg BID load for 2 doses 200 mg q12h	11 mg/kg q12h	Avoid i.v. formulation if CrCl is <50	Dose reduction for mild to moderate impairment	Primary aspergillosis Refractory *Scedosporium* and *Fusarium* infection Mucosal candidiasis Systemic candidiasis
Posaconazole	NA	No load 800 mg daily in divided doses	NA	No adjustments	No adjustments	Prophylaxis for invasive fungal infection Oropharyngeal candidiasis
Caspofungin	70-mg load 50 mg/day	NA	50 mg/m^2/day	No adjustments	35 mg/day for moderate impairment	Invasive candidiasis Candidemia Mucosal candidiasis Refractory aspergillosis
Micafungin	No load 100 mg/day	NA	1–4 mg/kg/day	No adjustments	No adjustments	Mucosal candidiasis Prophylaxis for invasive fungal infection
Anidulafungin	200 mg load 100 mg/day	NA	3-mg/kg load 1.5 mg/kg/day	No adjustments	No adjustments	Invasive candidiasis Candidemia Mucosal candidiasis

[a]NA, not applicable; q48h, every 48 h; BID, twice daily.

induction treatment for cryptococcal meningitis (35, 182, 222). Amphotericin B is recommended as initial therapy for treatment of the mycoses due to dimorphic fungi, including moderately severe to severe blastomycosis, histoplasmosis, and coccidioidomycosis (48, 87, 292). Although amphotericin B is approved for the treatment of aspergillosis, voriconazole has become the preferred therapy based on effectiveness in a large, multicenter trial (110). Finally, amphotericin B formulations remain the primary treatment option for emerging zygomycete infections (240).

FLUCYTOSINE

Mechanism of Action

Flucytosine is 5-fluorocytosine, a fluorinated pyrimidine which is related to fluorouracil and floxuridine (Fig. 1). It acts as a pyrimidine analog that is actively taken up by the fungal organism through a cytosine permease. It is then converted to fluorouracil by cytosine deaminase. The active metabolites of fluorouracil interfere with both DNA and RNA synthesis, ultimately inhibiting protein synthesis (280).

TABLE 5 Pharmacodynamic parameters for frequently used systemic antifungal agents

Agents	Pharmacodynamic index	Pharmacodynamic target	Pharmacodynamic character	PAFE
Polyenes	C_{max}/MIC	2–4	Concentration dependent	Prolonged
Flucytosine	Time above MIC	1.5	Time dependent	Short
Triazoles	AUC/MIC	25	Time dependent	Prolonged
Echinocandins	AUC/MIC	3–5	Concentration dependent	Prolonged

Spectrum of Activity

Flucytosine is active against most pathogenic yeasts but has minimal activity against filamentous fungi (Table 2). Flucytosine demonstrates in vitro activity against commonly isolated *Candida* spp., including *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* (Table 3). However, MICs are higher for *C. krusei* and *C. lusitaniae*. Flucytosine is active against *Cryptococcus* spp. (195). It does not demonstrate activity against the dimorphic fungi, *Aspergillus* spp., or the Zygomycetes group of fungi (6, 63). Use of flucytosine has been limited by the rapid emergence of resistant organisms, especially when used as monotherapy (176, 250).

Clinically Relevant Pharmacokinetics

Flucytosine is only available as an oral capsule and demonstrates excellent oral bioavailability (80 to 90%) (229). Flucytosine has a short half-life, less than 5 h, and thus requires fairly frequent dosing (four times daily) (62, 275). It is minimally bound to host proteins and has been shown to accumulate in most host tissues and fluids, including the cerebrospinal and intraocular fluids (62, 275).

Flucytosine is not metabolized, and greater than 90% of the drug is excreted in its native and microbiologically active form into the urine (75, 229). Therefore, a reduction in the dosing interval is required for patients with renal insufficiency and creatinine clearance (CrCl) of >50 (Table 4). Dose adjustments are not necessary for patients with hepatic insufficiency.

Pharmacodynamics

Several concentration-ranging, time-kill investigations have characterized the pharmacodynamic pattern of activity for flucytosine (Table 5) (11, 80, 114, 140, 262). The antifungal activity of flucytosine in both in vitro and in vivo *Candida* infection models has been shown to be maximal at concentrations not far above the MIC, and additional exposure to higher concentrations does not improve the extent of organism killing (i.e., time-dependent killing). In addition, these studies observed rapid recovery of *Candida* growth soon after drug exposure, consistent with the lack of or very short PAFEs. As predicted from these in vitro data, flucytosine dose fractionation studies in an in vivo candidiasis model showed that efficacy was optimal with dosing regimens in which flucytosine was administered frequently to maintain concentrations at or above the MIC (11). With the frequent dosing regimen that prolonged the time of the antifungal exposure, 10-fold less total drug was needed for efficacy. These results suggest that the %*T*>MIC would be the most predictive index associated with efficacy. The index magnitude for which optimal efficacy against *C. albicans* was noted in a mouse infection model was a %*T*>MIC magnitude of only 40% of the dosing interval, or concentrations in serum above the MIC for just less than half of the dosing interval (11, 114). There are limited clinical pharmacodynamic data available for corroboration of these observations.

The toxicodynamic relationships of flucytosine have also been well studied (85, 125, 179, 237, 242). Studies have found the primary toxicity of flucytosine, bone marrow toxicity, to be associated with high peak concentrations. The fact that the pharmacodynamic drivers of success and toxicity are different provides an opportunity to design dosing strategies to both optimize treatment efficacy and reduce toxicity.

Toxicities

The major toxicities of flucytosine include bone marrow suppression and hepatotoxicity. Bone marrow toxicity, including thrombocytopenia, may be severe and dose limiting (275). These adverse effects are most common with serum flucytosine concentrations of 125 μg/ml or greater (125). Patients with renal insufficiency are at an increased risk for toxicity and should be closely monitored. Other side effects of flucytosine include nausea, vomiting, diarrhea, and rash. Flucytosine is contraindicated during pregnancy due to the risk of fetal abnormalities identified in animal studies (158).

Drug-Drug Interactions

Flucytosine is not metabolized in the liver and is not a substrate or inhibitor of the CYP450 enzymes (Table 6). Therefore, few drug-drug interactions have been described.

Clinical Uses

Flucytosine is active against most *Candida* spp. but is rarely used alone for treatment of invasive candidiasis due to the rapid emergence of resistant subpopulations (176, 250). To prevent emergence of resistance, flucytosine is typically administered in combination with amphotericin B. This combination is a second- or third-line therapy for treatment of invasive candidiasis involving the central nervous system, eyes, or endovascular system. The single situation in which monotherapy may be sufficient is in treatment of isolated *Candida* bladder infection (180, 224, 275). While use in *Candida* infection is limited, the combination of flucytosine and amphotericin B is the first-line therapy for cryptococcal meningitis and has been shown to be superior to other antifungal therapies (35, 182, 222).

AZOLES

The introduction of the azole class of antifungal drugs began with the licensing of miconazole. Although systemic administration of the early azole formulations (miconazole and ketoconazole) was associated with significant toxicity, the newer agents (fluconazole, itraconazole, posaconazole, and voriconazole) (Fig. 1) are generally safe and well tolerated during systemic therapy (54). Miconazole and other azoles, including clotrimazole, butoconazole, tioconazole, and terconazole, are available as topical formulations for treatment of several cutaneous fungal syndromes and vaginal candidiasis (176).

TABLE 6 Common drug interactions for systemic antifungal agents

Agent	CYP450 interaction	Increases antifungal exposure	Decreases antifungal exposure	Antifungal known or likely to increase exposure	
				Contraindicated	Reduce dose and/or monitor
Amphotericin B	–	–	–	–	Digitalis glycosides Immunosuppressants
Flucytosine	–	–	Antimetabolites	Antiarrhythmics	–
Fluconazole	3A4 ++ 2C19 + 2C9 ++	–	Antibiotics	Antiarrhythmics Antipsychotics Immunosuppressants Migraine medications	Antibiotics Anticoagulants Antidepressants Antiseizure medications Antiretrovirals Blood pressure medications Chemotherapy agents Diabetes medications Immunosuppressants Hormonal therapy Lipid-lowering agents Narcotics Sedatives
Itraconazole	3A4 +++ 2C9 +	Antibiotics Antiretrovirals	Aluminum products Antacids Antibiotics Antiseizure medications Calcium products Magnesium products Sedatives	Antiarrhythmics Antipsychotics Immunosuppressants Lipid-lowering agents Migraine medications Sedatives	Antibiotics Anticoagulants Antidepressants Antiseizure medications Antiretrovirals Blood pressure medications Chemotherapy agents Diabetes medications Immunosuppressants Hormonal therapy Lipid-lowering agents Narcotics Proton pump inhibitors Sedatives
Voriconazole	3A4 ++ 2C19 +++ 2C9 ++	Antiretrovirals Hormonal therapy	Antibiotics Antiretrovirals Antiseizure medications Sedatives	Migraine medications Antipsychotics Antiarrhythmics Antibiotics Immunosuppressants Antianginal medications Supplements	Antibiotics Anticoagulants Antiseizure medications Blood pressure medications Chemotherapy agents Diabetes medications Hormonal therapy Immunosuppressants Lipid-lowering agents Narcotics Proton pump inhibitors Sedatives
Posaconazole	3A4 +++	–	Antacids Antibiotics Antiretrovirals Antiseizure medications	Antiarrhythmics Antipsychotics Migraine medications	Antiarrhythmics Antibiotics Antiretrovirals Antiseizure medications Blood pressure medications Chemotherapy agents Immunosuppressants Lipid-lowering agents Sedatives
Caspofungin	–	Immunosuppressants	Antibiotics Antiseizure medications Antiretrovirals Hormonal therapy	–	Immunosuppressants
Micafungin	–	–	–	–	Blood pressure medications Immunosuppressants
Anidulafungin	–	–	–	–	–

The antifungal azole drugs are classified chemically as imidazoles (clotrimazole, ketoconazole, and miconazole) or triazoles (fluconazole, itraconazole, voriconazole, and posaconazole) based on the number of nitrogen atoms (two or three, respectively) in the azole ring (220). Voriconazole, an expanded-spectrum triazole, was developed via systematic chemical manipulation of fluconazole to produce a compound with an enhanced potency and spectrum of activity. Posaconazole is chemically similar to itraconazole. The structures of both azoles contain extended piperazine-phenyl-triazole side chains, but posaconazole is composed of a furan ring with fluorine substituted for chlorine.

Mechanism of Action

The primary mode of action of azole drugs is inhibition of fungal CYP450 dependent C14-α sterol demethylase. Disruption of this critical step in synthesis of ergosterol, the major sterol of the fungal cell membrane, results in accumulation of sterol precursors and depletion of ergosterol (54, 109, 112, 161, 225, 267). The altered membrane permeability resulting from the change in sterol content halts fungal growth due to a variety of poorly defined effects on cell function. Unlike amphotericin B and the echinocandins, which are fungicidal against *Candida* spp., the azoles are fungistatic.

Spectrum of Activity

Fluconazole

Fluconazole exhibits activity against *Candida* spp. and is most active against *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, and *C. dubliniesis* (Table 3) (194). However, fluconazole has limited or no activity against *C. krusei,* and MICs are higher for *C. glabrata* and for several less commonly isolated *Candida* spp., including *C. guilliermondii* and *C. rugosa* (190). Fluconazole also demonstrates activity against *Cryptococcus neoformans* (187, 195) (Table 2). It is active against the dimorphic pathogens *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum*, although MICs are more than 10-fold higher than those of other expanded-spectrum triazoles such as itraconazole, posaconazole, and voriconazole (93). Fluconazole is not active against *Sporothrix schenckii* or most filamentous fungi, including *Aspergillus* spp., *Fusarium* spp., *Scedosporium* spp., and the zygomycetes (6, 61, 92, 93, 224, 247).

Itraconazole

Itraconazole exhibits a similar spectrum against *Candida* spp., but MICs are 10-fold lower for fluconazole (Table 3) (187, 189, 191). Like fluconazole MICs, itraconazole MICs are higher for *C. glabrata* and *C. krusei*. Itraconazole is also active against the dimorphic fungi *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Sporothrix schenckii*, and *Paracoccidioides* spp. but is less active against *Coccidioides immitis* (7, 93, 142, 151) (Table 2). Itraconazole exhibits activity against most *Aspergillus* spp., including *Aspergillus fumigatus*, *A. flavus*, *A. nidulans*, and *A. terreus*, but is less active against *A. niger* and *A. versicolor* (73). Itraconazole does not exhibit significant activity against the zygomycetes.

Voriconazole

The activity of voriconazole against *Candida* spp. is similar to that of fluconazole and itraconazole, but MICs are somewhat lower than those observed for fluconazole (189) (Table 3). Voriconazole is active against *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, and *C. dubliniensis* (186). In addition, it demonstrates enhanced efficacy against less commonly isolated *Candida* spp., including *C. krusei*, *C. lusitaniae*, *C. guilliermondii*, and *C. rugosa*, which are frequently resistant to the traditional antifungals amphotericin B and fluconazole (186, 190, 193). Voriconazole remains active against a subset of fluconazole-resistant strains (48%), although there is a trend of increasing MICs to voriconazole for strains with reduced fluconazole susceptibility (198, 200). The spectrum of voriconazole also includes *Cryptococcus neoformans* and the endemic fungal species *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum* (Table 2) (122, 142, 194, 195). Compared to earlier azoles, voriconazole is notable for potency against *Aspergillus* spp., including the polyene-resistant *A. terreus*, and the emerging and otherwise drug-resistant filamentous fungi *Fusarium* and *Scedosporium* (73, 93, 153). One notable hole in the spectrum against filamentous fungi is for the Zygomycetes group of organisms (188).

Posaconazole

Posaconazole also exhibits potent activity against commonly isolated *Candida* spp., with MICs 2- to 10-fold lower than those for itraconazole and fluconazole (187, 224) (Table 3). Posaconazole is active against less common and emerging *Candida* spp., including *C. dubliniensis*, *C. krusei*, *C. lusitaniae*, and *C. guilliermondii* (187, 190, 224). However, similar to those of fluconazole and itraconazole, MICs of posaconazole are higher for *C. glabrata* and *C. krusei*. Posaconazole remains active against a subset of fluconazole-resistant strains, although there is a strong relationship between increasing MICs of fluconazole and posaconazole (190). Posaconazole is active against *Cryptococcus* spp. and the dimorphic fungi *Histoplasma capsulatum*, *Blastomyces dermatitidis*, and *Coccidioides immitis* (93, 183, 187) (Table 2). However, posaconazole is less active against *Sporothrix schenckii* and *Paracoccidioides* spp., with MICs approximately twofold higher than those for itraconazole (93, 224, 246). Posaconazole exhibits activity against many filamentous fungi, and the most notable and unique aspect of posaconazole activity is against the several species from the emerging Zygomycetes group of fungi (73, 188). The spectrum of activity also includes *Aspergillus* spp., including *A. fumigatus*, *A. flavus*, *A. niger*, and the polyene-resistant *A. terreus* (73, 224).

Clinically Relevant Pharmacokinetics

The pharmacokinetic profiles of the azole drugs are distinct due to variation in molecular weight, aqueous solubility, and protein binding. Fluconazole is unique because of its relatively small size and high aqueous solubility. It has high oral bioavailability and effectively penetrates most body tissues and fluids, including the cerebrospinal fluid and ocular compartments. In contrast, the significantly lower aqueous solubility of voriconazole, itraconazole, and posaconazole hinders the oral bioavailability and complicates i.v. delivery of these drugs. Different approaches have been taken to ensure efficient and safe delivery of these lipophilic compounds into the systemic circulation. As one example, the oral solution and parenteral formulations of itraconazole have incorporated the azole in a carrier complex of hydroxypropyl-β-cyclodextrin, a large ring of substituted glucose molecules with a hydrophilic outer surface and a cylindrical hydrophobic inner core. A similar formulation was developed for the parenteral formulation of voriconazole.

Fluconazole

Fluconazole is supplied as an oral tablet, an oral powder for suspension, and as an i.v. solution. The bioavailability of fluconazole is near 90% and is not significantly affected by administration of food or by gastric acidity (136, 255, 300). Peak concentrations of fluconazole in plasma typically occur 2 to 3 h after administration and are proportional to the dose administered (54, 83). In the serum of healthy volunteers, fluconazole is approximately 11 to 13% protein bound (64). Fluconazole has been shown to penetrate most tissues, including the brain and eyes (94, 251, 281). Unlike the other triazole drugs, fluconazole is excreted into the urine in active form, with urine concentrations 10-fold greater than those in serum (84, 231). Fluconazole effectively accumulates in vaginal tissues, and a single dose of fluconazole (150 mg) has been shown to maintain therapeutic levels for more than 72 h (120). Fluconazole does not undergo significant metabolism and is excreted mostly unchanged in the urine. Therefore, the fluconazole dosage should be decreased in patients with renal impairment (Table 4). Hemodialysis and some forms of continuous hemofiltration deplete fluconazole from plasma, so fluconazole should be administered following hemodialysis (95, 159, 160, 265). Fluconazole does not require dose reduction for patients with hepatic impairment.

Itraconazole

Available preparations include an oral capsule and an oral solution (complexed with hydroxypropyl-β-cyclodextrin). Although not currently commercially available, itraconazole has also been formulated for i.v. administration. The bioavailability of itraconazole is dependent on the specific formulation and the gastric conditions. Itraconazole is lipophilic and poorly soluble in aqueous solutions at neutral pH. The absorption of the capsule formulation is near 55% but is enhanced by an acidic gastric environment, which can be produced by administration of an acidic beverage, such as Coca-Cola, and is diminished in patients receiving medications which suppress gastric acidity, such as proton pump inhibitors and histamine-2 blockers (75, 135). In addition, administration of the capsule formulation of itraconazole with food improves itraconazole bioavailability (269, 300). An oral solution using hydroxypropyl-β-cyclodextrin as a carrier molecule has the advantage of improved bioavailability, reaching 80% (118, 207, 244). Unlike with the capsule, food and gastric acidity do not improve the absorption of the oral solution formulation.

Itraconazole is approximately 99% protein bound, and total itraconazole levels in body fluids, including the vitreous and cerebrospinal fluids, are significantly lower than concentrations in plasma (227, 296). Itraconazole is distinct from fluconazole in that high concentrations accumulate in keratinous tissues (nearly 20-fold higher than in plasma), a characteristic favorable for treatment of skin and nail infections (65, 105, 111). Itraconazole is metabolized, primarily by the CYP450 isoenzyme 3A4, to at least three metabolites: hydroxyitraconazole, keto-itraconazole, and *N*-desalkyl-itraconazole. Itraconazole is unique because one of these metabolites, hydroxyitraconazole, exhibits antifungal activity similar to or greater than that of the parent compound (105). Elimination of these compounds follows saturable pharmacokinetics, with metabolites recovered in the feces and urine (54, 94, 105). However, the urinary metabolites are inactive (104). Although itraconazole dosage adjustments do not need to be made for patients with renal insufficiency, the hydroxypropyl-β-cyclodextrin vehicle of the intravenous preparation is renally cleared and high concentrations have been measured in patients with reduced kidney function. The oral solution can be used in patients with renal insufficiency because the carrier molecule is degraded in the gastrointestinal tract (244). Itraconazole is not removed by either hemodialysis or continuous ambulatory peritoneal dialysis (38). Itraconazole is hepatically metabolized; however, dose reduction is not recommended for patients with hepatic impairment (Table 4) (94, 105).

Voriconazole

Voriconazole is formulated as a tablet, an oral suspension, and a sulfobutyl-ether cyclodextrin solution for i.v. administration (Table 3). The bioavailabilities of the oral formulations of voriconazole are >90% and are optimal in the fasted state (75, 210). Gastric acid does not alter absorption of voriconazole. Serum voriconazole levels increase disproportionately with larger doses, suggesting the presence of saturable first-pass metabolism (208, 209). For example, increasing the voriconazole dose by a factor of 2 results in an approximately threefold increase in the concentration in serum (208). Initiation of therapy with the parenteral formulation and the administration of a higher dose for the first 24 h (loading regimen) help to ensure more rapid attainment of therapeutic concentrations (54, 122).

Voriconazole has a large volume of distribution and is 58% protein bound in the serum (122). Voriconazole penetrates well into the cerebrospinal fluid and eyes (>50% of concentrations in serum), similar to fluconazole, making therapy of disease in these sites an option (75, 106, 146, 253). The relatively rapid elimination half-life of 6 h warrants twice-daily dosing (Table 4). Voriconazole is hepatically metabolized by the CYP450 isoenzymes CYP2C9, CYP2C19, and CYP3A4 via N oxidation, with CYP2C19 as the major metabolic enzyme. Minimal active drug appears in the urine, limiting the use of this drug for therapy of fungal cystitis (2, 252). Voriconazole does require dose reduction for patients with mild to moderate hepatic impairment (2). The oral formulation does not require dose adjustment for renal insufficiency (Table 4). However, the cyclodextrin molecule which is utilized for the parenteral formulation is renally cleared. The consequences of plasma accumulation of cyclodextrin are uncertain, but use of the i.v. formulation in patients with reduced renal function (CrCl < 50) is not recommended. Voriconazole is not readily dialyzable, and dose adjustments are not required for patients receiving peritoneal dialysis, continuous venovenous hemodiafiltration, or hemodialysis (86, 181).

Posaconazole

Posaconazole is currently available only as an oral suspension. As with itraconazole capsules, the presence of food, especially a high-fat meal, significantly increases absorption of posaconazole (30, 32, 58). Similarly, absorption is reduced by coadministration of drugs that raise the gastric pH (3, 57, 132). The drug exhibits saturable absorption; thus, oral loading doses are not possible and the drug must be administered multiple times daily to produce therapeutic concentrations (56, 75). Steady-state levels are often not achieved for up to a week with posaconazole therapy, limiting its utility for early treatment of life-threatening fungal infections. The compound has a long elimination half-life (>24 h) and is highly protein bound (>98%). Although animal models have not demonstrated significant posaconazole concentra-

tions in the cerebrospinal fluid, posaconazole was effective in a rabbit meningitis model and success was observed in a small number of patients from an open-label trial of refractory central nervous system infections (183, 203). Modest vitreal penetration of posaconazole (21%) was reported for a single patient with a fungal eye infection (75, 241).

Posaconazole undergoes minimal metabolism and is primarily excreted unchanged into the bile and feces (130). Dosing adjustments are not required for patients with renal dysfunction, and posaconazole is not dialyzable (Table 4) (59). Pharmacokinetics have not been studied extensively in patients with hepatic insufficiency, and use is cautioned in patients with significant liver dysfunction.

Therapeutic Drug Monitoring

The pharmacokinetic variability of several azole drugs has prompted the consideration of therapeutic drug monitoring, or the measurement of serum drug concentrations with subsequent dosing regimen adjustment (31, 44, 104, 131, 137, 178, 205, 236, 285). For itraconazole, voriconazole, and posaconazole, a strong relationship between concentration in serum and efficacy has been established and accurate drug assays are available to clinicians. The following section describes monitoring data for these compounds.

Itraconazole

Several studies have correlated serum itraconazole concentration and treatment efficacy (36, 67, 68, 260). For itraconazole, the interpretation of the concentration is dependent upon the assay method. Measurements using high-performance liquid chromatography (HPLC) differentiate the active metabolite of itraconazole, hydroxyitraconazole, from the itraconazole parent compound (119). Bioassays are not able to make this distinction and routinely measure higher values (roughly five times) for itraconazole due to the additional activity of the metabolite (290). A number of large databases have examined the relationship between concentration and effect. One of the larger databases included 250 patients with oral candidiasis (218). The trough concentrations via HPLC associated with the highest success rate (83%) were >0.5 μg/ml. This value is lower than the value observed for success in treatment of invasive systemic fungal infections. For example, for a cohort with cryptococcal meningitis, treatment success was observed in 100% of patients with trough concentrations of >1 μg/ml. In contrast, only a partial clinical response was achieved in 66% of those patients with concentrations of <1 μg/ml (67). The concentration goal considered adequate for treatment of active fungal infections is 1 to 2 μg/ml using an HPLC assay. The target trough concentration most often suggested for prophylaxis is >0.5 μg/ml. The assay goal for the bioassay is roughly five times higher than for the HPLC assay.

Voriconazole

Common polymorphisms in the gene encoding the primary metabolic enzyme for voriconazole (CYP2C19) result in wide variability in systemic concentrations (70, 252). Patients with polymorphisms conferring poor voriconazole metabolism have higher drug concentrations and are at increased risk for toxicity (40, 236, 259, 283). Conversely, low serum voriconazole levels are observed in patients with polymorphisms resulting in extensive metabolism, and these lower concentrations have been associated with treatment failure (236). Several studies have linked voriconazole trough concentrations to treatment success for patients with invasive aspergillosis (22, 237). Improved clinical success and patient survival were observed in the cohort of patients with trough serum voriconazole concentrations ranging from 1 to 2 μg/ml. Adverse effects have been reported more commonly with concentrations exceeding 7 μg/ml.

Posaconazole

The wide patient-to-patient variability in serum posaconazole concentrations has been associated with erratic absorption (131, 285). Studies examining therapeutic drug monitoring for posaconazole are limited. In patients with aspergillosis, a correlation between serum posaconazole concentration and treatment outcome was observed in a salvage treatment trial. Efficacy was greatest in patients with steady-state concentrations of >1.25 μg/ml and intermediate in those with concentrations above 0.5 μg/ml (285). Additional studies are needed to define the optimal concentrations for treatment success.

Pharmacodynamics

In vitro and in vivo time-kill studies have been undertaken with all of the clinically available triazole compounds (14, 15, 17, 79, 80, 115, 145, 262, 289). These investigations have shown that triazoles exhibit growth inhibition at concentrations near the MIC. In addition, in vivo studies have observed prolonged growth suppression after levels in serum drop below the MIC. These prolonged in vivo PAFEs are hypothesized to be due to the profound sub-MIC activity of the azole drug class. Dose fractionation studies have demonstrated that efficacy is dependent upon the total dose (AUC) and is independent of the dosing frequency (145). This pattern, observed for all the triazole drugs, supports the 24-h AUC/MIC as the pharmacodynamic index linked to treatment efficacy (Table 5) (10, 14, 15).

Identifying the pharmacodynamic index magnitude associated with clinical success has been of interest due to the emergence of triazole resistance in *Candida* species. The wide MIC variation for triazoles is optimal for in vivo study, and the investigations have been more successful for triazoles than for amphotericin B. The 24-h AUC/MIC ratio necessary to produce efficacy is equal to a value near 25, the rough equivalent of providing the drug concentration near the organism MIC for 24 h (10, 14, 15, 18). This ratio target has been the same for organisms with widely distributed MICs, including those with high MICs due to differing mechanisms of resistance. This AUC/MIC ratio has also been similar among the triazoles, after accounting for the variability in protein binding by analyzing only the unbound or microbiologically active fraction of the antifungal.

Several patient data sets have been useful for examining the relationship among triazole dose, organism MIC, and clinical outcome (27, 50, 52, 173, 196, 218, 219). One study of over 1,000 patients with oropharyngeal candidiasis found optimum treatment efficacy with fluconazole exposures relative to the MIC of the infecting *Candida* species near a 24-h fluconazole dose/MIC (AUC/MIC) value of 25 (218). When the fluconazole AUC/MIC exceeded 25, clinical success was noted in 91 to 100% of patients. However, when AUC/MIC was less than 25, clinical failure occurred in 27 to 35% of patients. These findings and results of a similar analysis are congruent with data from the in vivo models (219). Together, the data were considered for the development of in vitro susceptibility breakpoints for fluconazole and other triazoles by the Clinical and Laboratory Standards Institute (CLSI). Analysis of fluconazole pharmacodynamics in patients with candidemia also suggests the utility of a pharmacodynamic target value of 24-h AUC/MIC greater than 25

(27, 50, 218, 249). Clinical analysis performed using data from over 400 patients in clinical trials treated with voriconazole and over 300 patients treated with itraconazole confirms this pharmacodynamic magnitude target when free drug concentrations are considered (196, 218). Similar data for posaconazole are not available.

Drug-Drug Interactions

Absorptions of two triazole formulations (itraconazole oral capsules and oral posaconazole) are affected by gastric acidity, as described in the pharmacokinetics section above. Therefore, medications that alter gastric pH, such as proton pump inhibitors and histamine-2 blockers, should be avoided (3, 57, 75, 132, 135). Triazole drugs interact with many other medications, mostly related to changes in hepatic metabolism, and a patient's prescription list should be reviewed carefully when instituting these antifungals. The azole drug interactions are primarily mediated via the hepatic CYP450 isoenzymes, and the extents to which individual isoenzymes (CYP2C19, CYP3A4, and CYP2C9) are affected vary among the triazole drugs (Table 6) (144). Fluconazole and posaconazole act as inhibitors of the CYP450 enzymes, while itraconazole and voriconazole are both inhibitors and substrates for these enzymes (41, 42, 236). As CYP450 inhibitors, other drugs metabolized by the same isoenzyme have the potential to reach toxic levels during coadministration with triazoles. However, if the triazole acts as a substrate for the isoenzyme, then antifungal metabolism and clearance may vary substantially if the triazole is coadministered with drugs known to inhibit or induce the isoenzyme. This may result in inadequate antifungal levels and therapeutic failure or elevated drug levels and increased toxicity.

Some of the most serious drug-drug interactions occur when triazoles are coadministered with sedatives, antiarrhythmics, antiseizure medications, immunosuppressants, blood pressure-lowering agents, and anticoagulants (Table 6). Many of these combinations are contraindicated due to the severity of the interactions. For other interacting drugs, dose adjustments and close monitoring for toxicities are often required. The lists of potential interactions for each of the triazoles are extensive. For example, the Micromedex Healthcare database lists 200 medications which have been observed or predicted to interact with itraconazole. The most severe implications of a failure to consider these potential interactions range from inadequate antifungal therapy to death associated with ventricular arrhythmia, organ failure, or life-threatening bleeding (138, 168, 261, 299).

Toxicities

If drug interactions are appropriately monitored, the triazole drugs are generally well tolerated. The most common adverse events include gastrointestinal complaints, rash, and headache (101, 234, 263). Voriconazole exhibits a unique reversible visual side effect (photopsia) in 30% of patients (110). The most clinically significant toxicity for itraconazole and voriconazole is a dose-related hepatotoxicity, which occurs in approximately 31 and 13% of patients, respectively. Although monitoring of liver function tests is recommended, the hepatotoxicity associated with azoles does not commonly require drug discontinuation (90). A photosensitive skin rash associated with voriconazole use has also been reported to occur in up to 7% of patients (110). Birth defects have been linked to administration of azoles during pregnancy, so azoles are contraindicated in pregnant women (158, 211).

Clinical Indications

Fluconazole

Fluconazole is indicated for treatment of both mucosal and systemic candidiasis. The studies demonstrating the effectiveness of fluconazole for treatment of invasive candidiasis have primarily involved nonneutropenic, immunocompromised patients (8, 133, 213, 217). In this patient cohort, fluconazole performs as well as amphotericin B or an echinocandin but is much better tolerated than the former. Although guidelines suggest fluconazole as an initial therapeutic option for invasive candidiasis, expert consensus does not recommend use for patients who are critically ill, neutropenic, or at risk for fluconazole-resistant isolates due to recent azole exposure (176). Numerous trials have compared fluconazole to other azole drugs and echinocandin drugs for the treatment of mucosal candidiasis of the mouth or esophagus (5, 71, 72, 97, 129, 202, 272, 277, 295). These studies have consistently found fluconazole effective for treatment of these diseases in both human immunodeficiency virus (HIV)-infected and non-HIV-infected patients. Considering the safety, relative effectiveness, and cost of fluconazole compared to those of more recently developed antifungal drugs, it remains the drug of choice for these disease states. Fluconazole is also a first-line agent for treatment of vulvovaginal candidiasis in nonpregnant women (176). In multiple trials, fluconazole has been compared to intravaginal azole therapy and oral itraconazole (170, 238, 268). These studies found single-dose fluconazole therapy to be well tolerated and at least as effective as intravaginal antifungal therapy for treatment of vaginal candidiasis (170, 204, 268). Complicated cases of vaginitis or recurrent vaginitis can be treated with fluconazole, but several doses may be required (239).

Fluconazole is approved for the management of cryptococcal infections and is primarily used for treatment of mild to moderate pulmonary disease, as consolidation therapy in patients with more severe cerebral disease who have responded to induction therapy, and for prevention of relapse (48, 206, 221, 222). Fluconazole is also a useful agent for prophylaxis of invasive fungal infection in high-risk patients. It is recommended for bone marrow transplant recipients with neutropenia and patients with chemotherapy-induced neutropenia (37, 176, 234). Although fluconazole has the disadvantage of not being active against *Aspergillus* and other filamentous fungi, it is significantly better tolerated than either amphotericin B or itraconazole (271). More recently, fluconazole has been compared to posaconazole for antifungal prophylaxis (55, 264). These studies have found posaconazole significantly more successful in preventing invasive aspergillosis and similar to fluconazole for prevention of candidiasis. Fluconazole has also been shown to be effective for prevention of fungal infection in other high-risk patient cohorts, including preterm infants, patients in intensive care units, and those undergoing liver transplantation (60, 149, 230, 232, 297).

Itraconazole

Itraconazole effectively treats oropharyngeal and esophageal candidiasis (235). The itraconazole oral solution has higher success rates than the capsules, presumably due to enhanced absorption of this formulation. In randomized trials of immunocompromised patients, fluconazole and the itraconazole oral solution result in similar endoscopic and clinical responses (97, 202, 295). However, itraconazole offers little advantage over fluconazole, and it is currently only

recommended for treatment of fluconazole-refractory esophageal and oropharyngeal candidiasis (43, 176, 201, 223). Comparative studies have also found itraconazole to be as effective as intravaginal azole therapies for the treatment of vaginal candidiasis (256, 257). A meta-analysis of six randomized controlled trials compared the efficacy and safety of itraconazole oral solution to those of fluconazole capsules for the treatment of uncomplicated acute vaginal and vulvovaginal candidiasis in nonpregnant women and reported no difference in outcome (204). Because itraconazole does not offer an advantage over single-dose fluconazole therapy, it is not recommended as a first-line treatment for uncomplicated vaginal candidiasis (176). Itraconazole has not been studied extensively for the treatment of candidemia or invasive candidiasis (270).

Itraconazole has been studied and shown to be useful for numerous other fungal species infections. It has been demonstrated to be effective in the management of several forms of *Aspergillus* infection. However, randomized trials examining the efficacy of itraconazole for primary therapy of invasive aspergillosis are lacking, and it is reserved for salvage therapy in patients refractory or intolerant to voriconazole and amphotericin B formulations (117, 243, 286). Itraconazole is approved for the treatment of several endemic fungal infections, including histoplasmosis, blastomycosis, coccidioidomycosis, paracoccidioidomycosis, and sporotrichosis. For the most part, it is reserved for mild to moderate disease without central nervous system involvement or as step-down therapy following treatment with amphotericin B (47, 48, 74, 88, 96, 143, 164, 174, 214, 215, 291, 292). Itraconazole is also first-line therapy for dermatophyte infection of the skin, fingernails, and toenails (66, 107, 124, 139, 248, 287).

Itraconazole has been shown to prevent invasive fungal infection in patients with hematologic malignancy or autologous bone marrow transplantation (91, 154, 166). However, it is associated with more gastrointestinal intolerance than other azole drugs, such as fluconazole, and is frequently discontinued due to side effects (271).

Voriconazole

Voriconazole has been shown to effectively treat both mucosal and systemic candidiasis (5, 133). A randomized, multicenter trial compared voriconazole to a regimen of amphotericin B followed by fluconazole for treatment of candidemia. The levels of success of the outcome were similar in the two groups, and voriconazole met noninferiority criteria. Voriconazole also has regulatory approval for treatment of esophageal candidiasis. In a randomized, double-blind, multicenter trial, the efficacy and safety of voriconazole were compared to those of fluconazole for treatment of esophageal candidiasis in immunocompromised patients (5). Voriconazole was found to be at least as effective as fluconazole. However, with few exceptions, it does not appear to offer a significant advantage over fluconazole for this indication. Its primary clinical use has been as step-down oral therapy for patients with infection due to *C. krusei* and fluconazole-resistant, voriconazole-susceptible *C. glabrata* (197).

Voriconazole is one of two compounds licensed in the United States for primary treatment of aspergillosis. The largest prospective, randomized trial for the treatment of invasive pulmonary aspergillosis demonstrated that voriconazole was superior to amphotericin B, with improved survival and overall response (110). Voriconazole is recommended for the primary treatment of invasive aspergillosis (286). It is also approved for treatment of refractory fungal infections caused by *Scedosporium* spp. or *Fusarium* spp. or for patients with intolerance of other antifungal therapy (184).

Posaconazole

Posaconazole is approved for treatment of oropharyngeal candidiasis. The available clinical data for posaconazole include reports of successful salvage therapy for azole-refractory oropharyngeal candidiasis in HIV-infected patients, and two comparisons showing efficacy comparable with that of fluconazole for esophageal candidiasis (233, 272, 274). The utility of posaconazole for treatment of candidemia and invasive candidiasis has not been established.

The role of posaconazole has been best characterized in the area of fungal infection prevention. Posaconazole is approved for prophylaxis of invasive fungal infection in hematopoietic stem cell transplant recipients with graft-versus-host disease and those with hematologic malignancies and neutropenia (55, 264). In a randomized trial of patients with neutropenia and hematologic malignancies comparing posaconazole to fluconazole or itraconazole prophylaxis, invasive fungal infection was diagnosed in fewer patients receiving posaconazole (2%) than in those receiving fluconazole or itraconazole (8%) (55). In addition, a significant survival benefit was found in favor of posaconazole. A second study compared posaconazole to fluconazole for antifungal prophylaxis in patients with graft-verus-host disease (264). Analysis of the primary endpoint, occurrence of invasive fungal infection, established posaconazole as noninferior to fluconazole (posaconazole, 5.3%; fluconazole, 9%). Although superiority of posaconazole was not confirmed, it was significantly more successful in preventing invasive aspergillosis.

Because of its broad spectrum of activity, posaconazole has been studied for treatment of many refractory fungal infections, including invasive aspergillosis, fusariosis, zygomycosis, histoplasmosis, and coccidioidomycosis (98, 212, 216, 245, 285). Posaconazole was approved in Europe for salvage treatment in patients with invasive aspergillosis who are refractory to amphotericin B or itraconazole. The overall success rate in an open-label trial was 42% for the posaconazole group and 26% for the control group (285). Although these study designs limit the statistical analyses, the results suggest that posaconazole may be useful in treatment of emerging diseases with poor prognoses, such as refractory zygomycosis, fusariosis, and various endemic fungal infections.

ECHINOCANDINS

The echinocandins are a novel class of semisynthetic lipopeptides and chemically are cyclic hexapeptides N linked to a fatty acyl side chain. Three echinocandins, caspofungin, micafungin, and anidulafungin, are currently available for the treatment and prevention of a variety of fungal infections.

Mechanism of Action

These molecules act by inhibiting synthesis of β-1,3 glucan, an essential polysaccharide in the cell walls of many pathogenic fungi (76). The echinocandins are fungicidal against *Candida* species but fungistatic against filamentous fungi, where they inhibit growth of the tip of fungal hyphae (39).

Spectrum of Activity

The spectra of activity for the echinocandins are sufficiently similar among the three available compounds to be considered interchangeable (Tables 2 and 3). Echinocandins

exhibit fungicidal activity against *C. albicans*, *C. glabrata*, *C. dubliensis*, *C. tropicalis*, and *C. krusei* (46, 192, 273). The *Candida* spp. for which this class demonstrates less in vitro activity are *C. parapsilosis* and *C. guilliermondii*, which are 50- to 100-fold less susceptible than other *Candida* species (Table 3) (29). This difference has raised concern that the latter species may be less responsive than other *Candida* spp. or require higher doses. However, the clinical relevance of the *C. parapsilosis* in vitro data has not yet been demonstrated in clinical trials. The echinocandins exhibit fungistatic activity against common *Aspergillus* spp. (82, 155, 163). They are generally not active against *Cryptococcus* spp., the Zygomycetes group, *Scedosporium* spp., or endemic fungal pathogens (Table 2) (6, 61, 73).

Clinically Relevant Pharmacokinetics

The echinocandins exhibit low oral bioavailability and are available only as parenteral agents (Table 3). With few exceptions, the pharmacological properties of the three compounds are comparable (75, 81, 108, 126). They have long half-lives (10 to 26 h) that allow for once-daily dosing. The echinocandins exhibit high protein binding (97 to near 100%) and do not achieve significant concentrations in the cerebrospinal fluid, eyes, or urine (75, 167). Use of these agents for treatment of fungal infections in these sites has not been extensively evaluated and should be cautioned. The major route of elimination for echinocandins is nonenzymatic degradation to inactive molecules which are excreted primarily in the bile (81). Anidulafungin undergoes slow chemical degradation at physiological temperature and pH to an inactive compound, with approximately 30% excreted in the feces and <1% in urine. Caspofungin is metabolized by hydrolysis and N acetylation, with 35% recovered in feces and 41% recovered in urine as biologically inactive degradation products. Micafungin is metabolized to M-1 (catechol form) by arylsulfatase, with further metabolism to the M-2 (methoxy form) by catechol-*o*-methyltransferase. Seventy-one percent of the dose is recovered in the feces, with an additional 11% of a microbiologically inactive molecule in the urine. None of the echinocandins are significant substrates for the CYP450 enzymes. Anidulafungin is unique in that it is not metabolized by the liver and is exclusively degraded in the plasma (273). Caspofungin is the only echinocandin for which dose reduction is recommended for patients with moderate to severe hepatic dysfunction (Table 4) (279). None of the echinocandins require dose adjustment for renal insufficiency or dialysis (77, 108).

Pharmacodynamics

In vitro and in vivo studies with echinocandin drugs have been performed using *Candida* models (13, 16, 20, 23, 51, 100, 102, 103, 116). Results from these investigations have consistently demonstrated a pattern of concentration-dependent killing effects and prolonged PAFEs. Similar to the polyene drug class, the extent of caspofungin killing of *C. albicans* varies substantially over only a small rise in concentration (78). In vivo studies have confirmed these pharmacodynamic characteristics. Providing large, infrequently administered doses of echinocandins has been shown to maximize efficacy (13, 20, 23, 293). The total amount of drug necessary to achieve various microbiological endpoints was approximately fivefold smaller when the dosing strategy employed large single doses rather than smaller, more frequent doses. This pharmacodynamic pattern suggests that either the C_{max}/MIC or 24-h total drug/MIC (AUC/MIC) would represent the driving pharmacodynamic index. The in vitro phenomenon of *Candida* being resistant to high concentrations of echinocandins has been termed the paradoxical effect and is suggested to be due to activation of the cell wall integrity pathway. This effect has not been shown clinically (294).

In vivo studies have been undertaken to determine the magnitude of the AUC/MIC index needed for treatment efficacy (20). The pharmacodynamic targets for the anidulafungin free drug (non-protein bound) 24-h AUC/MIC were from 10 to 20. Similar AUC/MIC targets were found for *C. albicans* and both micafungin and caspofungin (20, 23). However, additional studies undertaken with *C. glabrata* and *C. parapsilosis* unexpectedly observed a target that was two- to fourfold lower for these species than for *C. albicans* (9).

More recently clinical pharmacodynamic analyses have identified a similar relationship. A data set of over 500 patients from three candidemia trials provided the opportunity to examine the relationship among echinocandin pharmacokinetics, MIC, and treatment outcome (21). In patients with micafungin total drug AUC/MIC greater than 3,000, successful outcome was observed for 98%, while it was only observed in 84% of those with an AUC/MIC of <3,000. Considering the degree of protein binding for micafungin (>99%), this finding is consistent with data for *C. albicans* animal model studies. Of note, the AUC/MIC target of patients infected with *C. parapsilosis* was 10-fold lower than for the remaining cohort, a difference even larger than that observed in the animal model experiments. These findings corroborate animal model studies suggesting a difference in the pharmacodynamic target among *Candida* species.

Drug-Drug Interactions

Unlike the triazoles, the echinocandins are not major substrates, inducers, or inhibitors of the CYP450 enzymes, and thus, serious drug-drug interactions are uncommon (Table 6) (46, 75, 273). In vitro, caspofungin utilizes the OATP-1B1 transporter, which is also utilized by other drugs, including rifampin (226). A higher dosage of caspofungin should be administered if enzyme inducers, such as rifampin, phenytoin, or dexamethasone are coadministered (126). There are also potential interactions between the echinocandin agents and the immunosuppressants cyclosporine and tacrolimus. These interactions are not predicted to be severe. However, monitoring of these concomitant medications is recommended for patients receiving echinocandin therapy.

Toxicities

The echinocandins have excellent safety profiles and few, relatively rare side effects (46, 75, 273). The most commonly reported adverse effects include elevated liver aminotransferase enzyme levels, gastrointestinal upset, and headaches. Mild histamine-like symptoms have been observed in isolated cases during rapid echinocandin infusion.

Clinical Uses

Each of the echinocandins has been shown to successfully treat both mucosal and invasive candidiasis (24, 71, 72, 123, 129, 134, 157, 172, 175, 213, 276, 277). A multicenter, randomized trial comparing the efficacy of caspofungin to that of amphotericin B in the treatment of invasive candidiasis found caspofungin to be at least as effective and associated with significantly fewer adverse events (157). A similarly designed trial comparing micafungin to liposomal

amphotericin B for the treatment of candidemia and invasive candidiasis demonstrated nearly identical treatment success rates (89%) in each arm (134). When caspofungin and micafungin were compared directly, the two compounds were found to be equivalent (175). Furthermore, anidulafungin was found to be at least as effective as fluconazole for treatment of candidemia or invasive candidiasis (213). Based on the safety and effectiveness of the echinocandins, they are recommended as first-line options for initial therapy in adult patients with candidemia or invasive candidiasis (176). They are the preferred agents for initial therapy in patients with moderately severe to severe disease, those with previous exposure to azole, and patients with *C. glabrata* infections. Multiple randomized trials have also shown the echinocandins to be as effective as both amphotericin B and fluconazole for treatment of oropharyngeal and esophageal candidiasis (71, 72, 276, 277). However, due to the low cost and ease of administration, fluconazole is often used as first-line therapy for mucosal candidiasis.

Echinocandins have not been studied as primary therapy for aspergillosis. However, they demonstrate activity against *Aspergillus* spp. and are approved for patients with invasive aspergillosis who are refractory to or intolerant of other approved therapies (4, 128, 147, 148, 150, 286). This approval is based on retrospective analysis and salvage therapy trials (71, 147). A novel therapeutic strategy has been the combination of echinocandins with a mold-active triazole for aspergillosis. Results from experimental infection models suggest utility of this combination. Clinical study of this strategy is underway (150).

The echinocandins have also been shown to be efficacious in empirical treatment of fungal infection in patients with neutropenia who remain febrile despite antibacterial treatment (284). Compared to liposomal amphotericin B, caspofungin was better tolerated and more effective in treating baseline fungal infections. Additionally, micafungin is approved for prophylaxis in hematopoietic stem cell transplant recipients to prevent both *Candida* and *Aspergillus* infections based on a randomized, double-blind, multicenter trial comparing micafungin to fluconazole (266). Although trials have not studied each of the echinocandins for these indications, it is presumed that they will have equivalent efficacies, and they are most often used interchangeably (126).

CONCLUSIONS

Many of the available antifungal agents offer potent activity against *Candida* spp. and effectively treat superficial and invasive infections due to *Candida* spp. and other fungi. These agents differ greatly in terms of their pharmacokinetic properties, drug-drug interactions, and toxicities. Understanding these characteristics is important for safe administration and optimal efficacy.

The need for compounds with enhanced potencies and spectra of activity against non-*C. albicans Candida* spp. and other emerging filamentous fungi continues to drive the development of additional antifungal drugs. Other features that figure prominently on the "wish list" of these drugs in development include ideal pharmacokinetic features, such as oral and parenteral formulations; few drug interactions; a long elimination half-life, allowing for infrequent dosing; and limited toxicity. A promising new agent with a subset of these characteristics is isavuconazole, a triazole with a favorable pharmacokinetic profile (228, 289). Because it is a water-soluble drug with a long half-life, adequate levels in serum can be reached after oral or intravenous administration and the dosing interval can be extended. Clinical trials will be needed to assess the safety and efficacy of new agents, such as isavuconazole, to determine their clinical niche.

REFERENCES

1. **Albengres, E., H. Le Louet, and J. P. Tillement.** 1998. Systemic antifungal agents. Drug interactions of clinical significance. *Drug Saf.* **18:**83–97.
2. **Alffenaar, J. W., T. de Vos, D. R. Uges, and S. M. Daenen.** 2009. High voriconazole trough levels in relation to hepatic function: how to adjust the dosage? *Br. J. Clin. Pharmacol.* **67:**262–263.
3. **Alffenaar, J. W., S. van Assen, T. S. van der Werf, J. G. Kosterink, and D. R. Uges.** 2009. Omeprazole significantly reduces posaconazole serum trough level. *Clin. Infect. Dis.* **48:**839.
4. **Aliff, T. B., P. G. Maslak, J. G. Jurcic, M. L. Heaney, K. N. Cathcart, K. A. Sepkowitz, and M. A. Weiss.** 2003. Refractory *Aspergillus* pneumonia in patients with acute leukemia: successful therapy with combination caspofungin and liposomal amphotericin. *Cancer* **97:**1025–1032.
5. **Ally, R., D. Schurmann, W. Kreisel, G. Carosi, K. Aguirrebengoa, B. Dupont, M. Hodges, P. Troke, and A. J. Romero.** 2001. A randomized, double-blind, double-dummy, multicenter trial of voriconazole and fluconazole in the treatment of esophageal candidiasis in immunocompromised patients. *Clin. Infect. Dis.* **33:**1447–1454.
6. **Almyroudis, N. G., D. A. Sutton, A. W. Fothergill, M. G. Rinaldi, and S. Kusne.** 2007. In vitro susceptibilities of 217 clinical isolates of zygomycetes to conventional and new antifungal agents. *Antimicrob. Agents Chemother.* **51:** 2587–2590.
7. **Alvarado-Ramirez, E., and J. M. Torres-Rodriguez.** 2007. In vitro susceptibility of *Sporothrix schenckii* to six antifungal agents determined using three different methods. *Antimicrob. Agents Chemother.* **51:**2420–2423.
8. **Anaissie, E. J., R. O. Darouiche, D. Abi-Said, O. Uzun, J. Mera, L. O. Gentry, T. Williams, D. P. Kontoyiannis, C. L. Karl, and G. P. Bodey.** 1996. Management of invasive candidal infections: results of a prospective, randomized, multicenter study of fluconazole versus amphotericin B and review of the literature. *Clin. Infect. Dis.* **23:**964–972.
9. **Andes, D., D. J. Diekema, M. A. Pfaller, J. Bohrmuller, K. Marchillo, and A. Lepak.** 2010. In vivo comparison of the pharmacodynamic targets for echinocandin drugs against *Candida* species. *Antimicrob. Agents Chemother.* **54:** 2497–2506.
10. **Andes, D., and M. van Ogtrop.** 1999. Characterization and quantitation of the pharmacodynamics of fluconazole in a neutropenic murine disseminated candidiasis infection model. *Antimicrob. Agents Chemother.* **43:**2116–2120.
11. **Andes, D., and M. van Ogtrop.** 2000. In vivo characterization of the pharmacodynamics of flucytosine in a neutropenic murine disseminated candidiasis model. *Antimicrob. Agents Chemother.* **44:**938–942.
12. **Andes, D., T. Stamsted, and R. Conklin.** 2001. Pharmacodynamics of amphotericin B in a neutropenic-mouse disseminated-candidiasis model. *Antimicrob. Agents Chemother.* **45:**922–926.
13. **Andes, D., K. Marchillo, J. Lowther, A. Bryskier, T. Stamstad, and R. Conklin.** 2003. In vivo pharmacodynamics of HMR 3270, a glucan synthase inhibitor, in a murine candidiasis model. *Antimicrob. Agents Chemother.* **47:** 1187–1192.
14. **Andes, D., K. Marchillo, T. Stamstad, and R. Conklin.** 2003. In vivo pharmacokinetics and pharmacodynamics of

a new triazole, voriconazole, in a murine candidiasis model. *Antimicrob. Agents Chemother.* **47:**3165–3169.
15. **Andes, D., K. Marchillo, R. Conklin, G. Krishna, F. Ezzet, A. Cacciapuoti, and D. Loebenberg.** 2004. Pharmacodynamics of a new triazole, posaconazole, in a murine model of disseminated candidiasis. *Antimicrob. Agents Chemother.* **48:**137–142.
16. **Andes, D., and N. Safdar.** 2005. Efficacy of micafungin for the treatment of candidemia. *Eur. J. Clin. Microbiol. Infect. Dis.* **24:**662–664.
17. **Andes, D., A. Forrest, A. Lepak, J. Nett, K. Marchillo, and L. Lincoln.** 2006. Impact of antimicrobial dosing regimen on evolution of drug resistance in vivo: fluconazole and *Candida albicans*. *Antimicrob. Agents Chemother.* **50:** 2374–2383.
18. **Andes, D., A. Lepak, J. Nett, L. Lincoln, and K. Marchillo.** 2006. In vivo fluconazole pharmacodynamics and resistance development in a previously susceptible *Candida albicans* population examined by microbiologic and transcriptional profiling. *Antimicrob. Agents Chemother.* **50:**2384–2394.
19. **Andes, D., N. Safdar, K. Marchillo, and R. Conklin.** 2006. Pharmacokinetic-pharmacodynamic comparison of amphotericin B (AMB) and two lipid-associated AMB preparations, liposomal AMB and AMB lipid complex, in murine candidiasis models. *Antimicrob. Agents Chemother.* **50:**674–684.
20. **Andes, D., D. J. Diekema, M. A. Pfaller, R. A. Prince, K. Marchillo, J. Ashbeck, and J. Hou.** 2008. In vivo pharmacodynamic characterization of anidulafungin in a neutropenic murine candidiasis model. *Antimicrob. Agents Chemother.* **52:**539–550.
21. **Andes, D., P. G. Ambrose, J. P. Hammel, S. A. Van Wart, V. Iyer, D. K. Reynolds, D. N. Buell, L. L. Kovanda, and S. M. Bhavnani.** 2011. Use of pharmacokinetic-pharmacodynamic analyses to optimize therapy with the systemic antifungal micafungin for invasive candidiasis or candidemia. *Antimicrob. Agents Chemother.* **55:**2113–2121.
22. **Andes, D., A. Pascual, and O. Marchetti.** 2009. Antifungal therapeutic drug monitoring: established and emerging indications. *Antimicrob. Agents Chemother.* **53:**24–34.
23. **Andes, D. R., D. J. Diekema, M. A. Pfaller, K. Marchillo, and J. Bohrmueller.** 2008. In vivo pharmacodynamic target investigation for micafungin against *Candida albicans* and *C. glabrata* in a neutropenic murine candidiasis model. *Antimicrob. Agents Chemother.* **52:**3497–3503.
24. **Arathoon, E. G., E. Gotuzzo, L. M. Noriega, R. S. Berman, M. J. DiNubile, and C. A. Sable.** 2002. Randomized, double-blind, multicenter study of caspofungin versus amphotericin B for treatment of oropharyngeal and esophageal candidiases. *Antimicrob. Agents Chemother.* **46:** 451–457.
25. **Arikan, S., and J. H. Rex.** 2001. Lipid-based antifungal agents: current status. *Curr. Pharm. Des.* **7:**393–415.
26. **Atkinson, A. J., Jr., and J. E. Bennett.** 1978. Amphotericin B pharmacokinetics in humans. *Antimicrob. Agents Chemother.* **13:**271–276.
27. **Baddley, J. W., M. Patel, S. M. Bhavnani, S. A. Moser, and D. R. Andes.** 2008. Association of fluconazole pharmacodynamics with mortality in patients with candidemia. *Antimicrob. Agents Chemother.* **52:**3022–3028.
28. **Baddley, J. W., K. A. Marr, D. R. Andes, T. J. Walsh, C. A. Kauffman, D. P. Kontoyiannis, J. I. Ito, S. A. Balajee, P. G. Pappas, and S. A. Moser.** 2009. Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the Transplant-Associated Infection Surveillance Network. *J. Clin. Microbiol.* **47:**3271–3275.
29. **Barchiesi, F., E. Spreghini, S. Tomassetti, A. Della Vittoria, D. Arzeni, E. Manso, and G. Scalise.** 2006. Effects of caspofungin against *Candida guilliermondii* and *Candida parapsilosis*. *Antimicrob. Agents Chemother.* **50:**2719–2727.
30. **Barone, J. A., J. G. Koh, R. H. Bierman, J. L. Colaizzi, K. A. Swanson, M. C. Gaffar, B. L. Moskovitz, W. Mechlinski, and V. Van de Velde.** 1993. Food interaction and steady-state pharmacokinetics of itraconazole capsules in healthy male volunteers. *Antimicrob. Agents Chemother.* **37:**778–784.
31. **Barone, J. A., B. L. Moskovitz, J. Guarnieri, A. E. Hassell, J. L. Colaizzi, R. H. Bierman, and L. Jessen.** 1998. Enhanced bioavailability of itraconazole in hydroxypropyl-beta-cyclodextrin solution versus capsules in healthy volunteers. *Antimicrob. Agents Chemother.* **42:**1862–1865.
32. **Barone, J. A., B. L. Moskovitz, J. Guarnieri, A. E. Hassell, J. L. Colaizzi, R. H. Bierman, and L. Jessen.** 1998. Food interaction and steady-state pharmacokinetics of itraconazole oral solution in healthy volunteers. *Pharmacotherapy* **18:**295–301.
33. **Bates, D. W., L. Su, D. T. Yu, G. M. Chertow, D. L. Seger, D. R. Gomes, E. J. Dasbach, and R. Platt.** 2001. Mortality and costs of acute renal failure associated with amphotericin B therapy. *Clin. Infect. Dis.* **32:**686–693.
34. **Bekersky, I., R. M. Fielding, D. E. Dressler, J. W. Lee, D. N. Buell, and T. J. Walsh.** 2002. Pharmacokinetics, excretion, and mass balance of liposomal amphotericin B (AmBisome) and amphotericin B deoxycholate in humans. *Antimicrob. Agents Chemother.* **46:**828–833.
35. **Bennett, J. E., W. E. Dismukes, R. J. Duma, G. Medoff, M. A. Sande, H. Gallis, J. Leonard, B. T. Fields, M. Bradshaw, H. Haywood, Z. A. McGee, T. R. Cate, C. G. Cobbs, J. F. Warner, and D. W. Alling.** 1979. A comparison of amphotericin B alone and combined with flucytosine in the treatment of cryptococcal meningitis. *N. Engl. J. Med.* **301:**126–131.
36. **Berenguer, J., N. M. Ali, M. C. Allende, J. Lee, K. Garrett, S. Battaglia, S. C. Piscitelli, M. G. Rinaldi, P. A. Pizzo, and T. J. Walsh.** 1994. Itraconazole for experimental pulmonary aspergillosis: comparison with amphotericin B, interaction with cyclosporin A, and correlation between therapeutic response and itraconazole concentrations in plasma. *Antimicrob. Agents Chemother.* **38:** 1303–1308.
37. **Bodey, G. P., E. J. Anaissie, L. S. Elting, E. Estey, S. O'Brien, and H. Kantarjian.** 1994. Antifungal prophylaxis during remission induction therapy for acute leukemia fluconazole versus intravenous amphotericin B. *Cancer* **73:**2099–2106.
38. **Boelaert, J., M. Schurgers, E. Matthys, R. Daneels, A. van Peer, K. De Beule, R. Woestenborghs, and J. Heykants.** 1988. Itraconazole pharmacokinetics in patients with renal dysfunction. *Antimicrob. Agents Chemother.* **32:** 1595–1597.
39. **Bowman, J. C., P. S. Hicks, M. B. Kurtz, H. Rosen, D. M. Schmatz, P. A. Liberator, and C. M. Douglas.** 2002. The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. *Antimicrob. Agents Chemother.* **46:**3001–3012.
40. **Boyd, A. E., S. Modi, S. J. Howard, C. B. Moore, B. G. Keevil, and D. W. Denning.** 2004. Adverse reactions to voriconazole. *Clin. Infect. Dis.* **39:**1241–1244.
41. **Bruggemann, R. J., J. W. Alffenaar, N. M. Blijlevens, E. M. Billaud, J. G. Kosterink, P. E. Verweij, and D. M. Burger.** 2009. Clinical relevance of the pharmacokinetic interactions of azole antifungal drugs with other coadministered agents. *Clin. Infect. Dis.* **48:**1441–1458.
42. **Brüggemann, R. J. M., J. C. Alffenaar, N. M. A. Blijlevens, E. M. Billaud, J. G. W. Kosterink, P. E. Verweij, and D. M. Burger.** 2008. Pharmacokinetic drug interactions of azoles. *Curr. Fungal Infect. Rep.* **2:**20–27.

43. **Cartledge, J. D., J. Midgley, M. Youle, and B. G. Gazzard.** 1994. Itraconazole cyclodextrin solution—effective treatment for HIV-related candidosis unresponsive to other azole therapy. *J. Antimicrob. Chemother.* **33:**1071–1073.
44. **Cartledge, J. D., J. Midgely, and B. G. Gazzard.** 1997. Itraconazole solution: higher serum drug concentrations and better clinical response rates than the capsule formulation in acquired immunodeficiency syndrome patients with candidosis. *J. Clin. Pathol.* **50:**477–480.
45. **Casado, J. L., C. Quereda, J. Oliva, E. Navas, A. Moreno, V. Pintado, J. Cobo, and I. Corral.** 1997. Candidal meningitis in HIV-infected patients: analysis of 14 cases. *Clin. Infect. Dis.* **25:**673–676.
46. **Chandrasekar, P. H., and J. D. Sobel.** 2006. Micafungin: a new echinocandin. *Clin. Infect. Dis.* **42:**1171–1178.
47. **Chapman, S. W., R. W. Bradsher, Jr., G. D. Campbell, Jr., P. G. Pappas, and C. A. Kauffman.** 2000. Practice guidelines for the management of patients with blastomycosis. Infectious Diseases Society of America. *Clin. Infect. Dis.* **30:**679–683.
48. **Chapman, S. W., W. E. Dismukes, L. A. Proia, R. W. Bradsher, P. G. Pappas, M. G. Threlkeld, and C. A. Kauffman.** 2008. Clinical practice guidelines for the management of blastomycosis: 2008 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **46:** 1801–1812.
49. **Cheng, J. T., R. T. Witty, R. R. Robinson, and W. E. Yarger.** 1982. Amphotericin B nephrotoxicity: increased renal resistance and tubule permeability. *Kidney Int.* **22:**626–633.
50. **Clancy, C. J., V. L. Yu, A. J. Morris, D. R. Snydman, and M. H. Nguyen.** 2005. Fluconazole MIC and the fluconazole dose/MIC ratio correlate with therapeutic response among patients with candidemia. *Antimicrob. Agents Chemother.* **49:**3171–3177.
51. **Clancy, C. J., H. Huang, S. Cheng, H. Derendorf, and M. H. Nguyen.** 2006. Characterizing the effects of caspofungin on *Candida albicans*, *Candida parapsilosis*, and *Candida glabrata* isolates by simultaneous time-kill and postantifungal-effect experiments. *Antimicrob. Agents Chemother.* **50:**2569–2572.
52. **Clancy, C. J., B. Staley, and M. H. Nguyen.** 2006. In vitro susceptibility of breakthrough *Candida* bloodstream isolates correlates with daily and cumulative doses of fluconazole. *Antimicrob. Agents Chemother.* **50:**3496–3498.
53. **Clements, J. S., Jr., and J. E. Peacock, Jr.** 1990. Amphotericin B revisited: reassessment of toxicity. *Am. J. Med.* **88**:22N–27N.
54. **Como, J. A., and W. E. Dismukes.** 1994. Oral azole drugs as systemic antifungal therapy. *N. Engl. J. Med.* **330:** 263–272.
55. **Cornely, O. A., J. Maertens, D. J. Winston, J. Perfect, A. J. Ullmann, T. J. Walsh, D. Helfgott, J. Holowiecki, D. Stockelberg, Y. T. Goh, M. Petrini, C. Hardalo, R. Suresh, and D. Angulo-Gonzalez.** 2007. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. *N. Engl. J. Med.* **356:**348–359.
56. **Courtney, R., S. Pai, M. Laughlin, J. Lim, and V. Batra.** 2003. Pharmacokinetics, safety, and tolerability of oral posaconazole administered in single and multiple doses in healthy adults. *Antimicrob. Agents Chemother.* **47:**2788–2795.
57. **Courtney, R., E. Radwanski, J. Lim, and M. Laughlin.** 2004. Pharmacokinetics of posaconazole coadministered with antacid in fasting or nonfasting healthy men. *Antimicrob. Agents Chemother.* **48:**804–808.
58. **Courtney, R., D. Wexler, E. Radwanski, J. Lim, and M. Laughlin.** 2004. Effect of food on the relative bioavailability of two oral formulations of posaconazole in healthy adults. *Br. J. Clin. Pharmacol.* **57:**218–222.
59. **Courtney, R., A. Sansone, W. Smith, T. Marbury, P. Statkevich, M. Martinho, M. Laughlin, and S. Swan.** 2005. Posaconazole pharmacokinetics, safety, and tolerability in subjects with varying degrees of chronic renal disease. *J. Clin. Pharmacol.* **45:**185–192.
60. **Cruciani, M., F. de Lalla, and C. Mengoli.** 2005. Prophylaxis of *Candida* infections in adult trauma and surgical intensive care patients: a systematic review and meta-analysis. *Intensive Care Med.* **31:**1479–1487.
61. **Cuenca-Estrella, M., B. Ruiz-Diez, J. V. Martinez-Suarez, A. Monzon, and J. L. Rodriguez-Tudela.** 1999. Comparative in-vitro activity of voriconazole (UK-109,496) and six other antifungal agents against clinical isolates of *Scedosporium prolificans* and *Scedosporium apiospermum*. *J. Antimicrob. Chemother.* **43:**149–151.
62. **Cutler, R. E., A. D. Blair, and M. R. Kelly.** 1978. Flucytosine kinetics in subjects with normal and impaired renal function. *Clin. Pharmacol. Ther.* **24:**333–342.
63. **Dannaoui, E., J. Meletiadis, J. W. Mouton, J. F. Meis, and P. E. Verweij.** 2003. In vitro susceptibilities of zygomycetes to conventional and new antifungals. *J. Antimicrob. Chemother.* **51:**45–52.
64. **Debruyne, D.** 1997. Clinical pharmacokinetics of fluconazole in superficial and systemic mycoses. *Clin. Pharmacokinet.* **33:**52–77.
65. **De Doncker, P., J. Decroix, G. E. Pierard, D. Roelant, R. Woestenborghs, P. Jacqmin, F. Odds, A. Heremans, P. Dockx, and D. Roseeuw.** 1996. Antifungal pulse therapy for onychomycosis. A pharmacokinetic and pharmacodynamic investigation of monthly cycles of 1-week pulse therapy with itraconazole. *Arch. Dermatol.* **132:**34–41.
66. **Degreef, H., K. Marien, H. De Veylder, K. Duprez, A. Borghys, and L. Verhoeve.** 1987. Itraconazole in the treatment of dermatophytoses: a comparison of two daily dosages. *Rev. Infect. Dis.* **9**(Suppl. 1)**:**S104–S108.
67. **Denning, D. W., R. M. Tucker, L. H. Hanson, J. R. Hamilton, and D. A. Stevens.** 1989. Itraconazole therapy for cryptococcal meningitis and cryptococcosis. *Arch. Intern. Med.* **149:**2301–2308.
68. **Denning, D. W., R. M. Tucker, L. H. Hanson, and D. A. Stevens.** 1989. Treatment of invasive aspergillosis with itraconazole. *Am. J. Med.* **86:**791–800.
69. **Denning, D. W., K. A. Marr, W. M. Lau, D. P. Facklam, V. Ratanatharathorn, C. Becker, A. J. Ullmann, N. L. Seibel, P. M. Flynn, J. A. van Burik, D. N. Buell, and T. F. Patterson.** 2006. Micafungin (FK463), alone or in combination with other systemic antifungal agents, for the treatment of acute invasive aspergillosis. *J. Infect.* **53:** 337–349.
70. **Desta, Z., X. Zhao, J. G. Shin, and D. A. Flockhart.** 2002. Clinical significance of the cytochrome P450 2C19 genetic polymorphism. *Clin. Pharmacokinet.* **41:**913–958.
71. **de Wet, N., A. Llanos-Cuentas, J. Suleiman, E. Baraldi, E. F. Krantz, M. Della Negra, and H. Diekmann-Berndt.** 2004. A randomized, double-blind, parallel-group, dose-response study of micafungin compared with fluconazole for the treatment of esophageal candidiasis in HIV-positive patients. *Clin. Infect. Dis.* **39:**842–849.
72. **de Wet, N. T., A. J. Bester, J. J. Viljoen, F. Filho, J. M. Suleiman, E. Ticona, E. A. Llanos, C. Fisco, W. Lau, and D. Buell.** 2005. A randomized, double blind, comparative trial of micafungin (FK463) vs. fluconazole for the treatment of oesophageal candidiasis. *Aliment. Pharmacol. Ther.* **21:**899–907.
73. **Diekema, D. J., S. A. Messer, R. J. Hollis, R. N. Jones, and M. A. Pfaller.** 2003. Activities of caspofungin, itraconazole, posaconazole, ravuconazole, voriconazole, and amphotericin B against 448 recent clinical isolates of filamentous fungi. *J. Clin. Microbiol.* **41:**3623–3626.

74. **Dismukes, W. E., R. W. Bradsher, Jr., G. C. Cloud, C. A. Kauffman, S. W. Chapman, R. B. George, D. A. Stevens, W. M. Girard, M. S. Saag, and C. Bowles-Patton.** 1992. Itraconazole therapy for blastomycosis and histoplasmosis. NIAID Mycoses Study Group. *Am. J. Med.* **93:** 489–497.
75. **Dodds Ashley, E. S., R. Lewis, J. S. Lewis, C. Martin, and D. Andes.** 2006. Pharmacology of systemic antifungal agents. *Clin. Infect. Dis.* **43:**S28–S39.
76. **Douglas, C. M., J. A. D'Ippolito, G. J. Shei, M. Meinz, J. Onishi, J. A. Marrinan, W. Li, G. K. Abruzzo, A. Flattery, K. Bartizal, A. Mitchell, and M. B. Kurtz.** 1997. Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1,3-beta-D-glucan synthase inhibitors. *Antimicrob. Agents Chemother.* **41:**2471–2479.
77. **Dowell, J. A., M. Stogniew, D. Krause, and B. Damle.** 2007. Anidulafungin does not require dosage adjustment in subjects with varying degrees of hepatic or renal impairment. *J. Clin. Pharmacol.* **47:**461–470.
78. **Ernst, E. J., M. E. Klepser, M. E. Ernst, S. A. Messer, and M. A. Pfaller.** 1999. In vitro pharmacodynamic properties of MK-0991 determined by time-kill methods. *Diagn. Microbiol. Infect. Dis.* **33:**75–80.
79. **Ernst, E. J., M. E. Klepser, and M. A. Pfaller.** 2000. Postantifungal effects of echinocandin, azole, and polyene antifungal agents against *Candida albicans* and *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.* **44:**1108–1111.
80. **Ernst, E. J., K. Yodoi, E. E. Roling, and M. E. Klepser.** 2002. Rates and extents of antifungal activities of amphotericin B, flucytosine, fluconazole, and voriconazole against *Candida lusitaniae* determined by microdilution, Etest, and time-kill methods. *Antimicrob. Agents Chemother.* **46:**578–581.
81. **Eschenauer, G., D. D. Depestel, and P. L. Carver.** 2007. Comparison of echinocandin antifungals. *Ther. Clin. Risk Manag.* **3:**71–97.
82. **Espinel-Ingroff, A.** 1998. Comparison of In vitro activities of the new triazole SCH56592 and the echinocandins MK-0991 (L-743,872) and LY303366 against opportunistic filamentous and dimorphic fungi and yeasts. *J. Clin. Microbiol.* **36:**2950–2956.
83. **Ezzet, F., D. Wexler, R. Courtney, G. Krishna, J. Lim, and M. Laughlin.** 2005. Oral bioavailability of posaconazole in fasted healthy subjects: comparison between three regimens and basis for clinical dosage recommendations. *Clin. Pharmacokinet.* **44:**211–220.
84. **Foulds, G., C. Wajszczuk, D. J. Weidler, D. J. Garg, and P. Gibson.** 1988. Steady state parenteral kinetics of fluconazole in man. *Ann. N. Y. Acad. Sci.* **544:**427–430.
85. **Francis, P., and T. J. Walsh.** 1992. Evolving role of flucytosine in immunocompromised patients: new insights into safety, pharmacokinetics, and antifungal therapy. *Clin. Infect. Dis.* **15:**1003–1018.
86. **Fuhrmann, V., P. Schenk, W. Jaeger, M. Miksits, N. Kneidinger, J. Warszawska, U. Holzinger, R. Kitzberger, and F. Thalhammer.** 2007. Pharmacokinetics of voriconazole during continuous venovenous haemodiafiltration. *J. Antimicrob. Chemother.* **60:**1085–1090.
87. **Galgiani, J. N., N. M. Ampel, A. Catanzaro, R. H. Johnson, D. A. Stevens, and P. L. Williams.** 2000. Practice guideline for the treatment of coccidioidomycosis. Infectious Diseases Society of America. *Clin. Infect. Dis.* **30:**658–661.
88. **Galgiani, J. N., N. M. Ampel, J. E. Blair, A. Catanzaro, R. H. Johnson, D. A. Stevens, and P. L. Williams.** 2005. Coccidioidomycosis. *Clin. Infect. Dis.* **41:**1217–1223.
89. **Gallis, H. A., R. H. Drew, and W. W. Pickard.** 1990. Amphotericin B: 30 years of clinical experience. *Rev. Infect. Dis.* **12:**308–329.
90. **Girois, S. B., F. Chapuis, E. Decullier, and B. G. Revol.** 2005. Adverse effects of antifungal therapies in invasive fungal infections: review and meta-analysis. *Eur. J. Clin. Microbiol. Infect. Dis.* **24:**119–130.
91. **Glasmacher, A., E. Molitor, C. Hahn, K. Bomba, S. Ewig, C. Leutner, E. Wardelmann, I. G. Schmidt-Wolf, J. Mezger, G. Marklein, and T. Sauerbruch.** 1998. Antifungal prophylaxis with itraconazole in neutropenic patients with acute leukaemia. *Leukemia* **12:**1338–1343.
92. **Gonzalez, G. M., D. A. Sutton, E. Thompson, R. Tijerina, and M. G. Rinaldi.** 2001. In vitro activities of approved and investigational antifungal agents against 44 clinical isolates of basidiomycetous fungi. *Antimicrob. Agents Chemother.* **45:**633–635.
93. **Gonzalez, G. M., A. W. Fothergill, D. A. Sutton, M. G. Rinaldi, and D. Loebenberg.** 2005. In vitro activities of new and established triazoles against opportunistic filamentous and dimorphic fungi. *Med. Mycol.* **43:**281–284.
94. **Grant, S. M., and S. P. Clissold.** 1989. Itraconazole. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in superficial and systemic mycoses. *Drugs* **37:**310–344.
95. **Grant, S. M., and S. P. Clissold.** 1990. Fluconazole. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in superficial and systemic mycoses. *Drugs* **39:**877–916.
96. **Graybill, J. R., D. A. Stevens, J. N. Galgiani, W. E. Dismukes, and G. A. Cloud.** 1990. Itraconazole treatment of coccidioidomycosis. NAIAD Mycoses Study Group. *Am. J. Med.* **89:**282–290.
97. **Graybill, J. R., J. Vazquez, R. O. Darouiche, R. Morhart, D. Greenspan, C. Tuazon, L. J. Wheat, J. Carey, I. Leviton, R. G. Hewitt, R. R. MacGregor, W. Valenti, M. Restrepo, and B. L. Moskovitz.** 1998. Randomized trial of itraconazole oral solution for oropharyngeal candidiasis in HIV/AIDS patients. *Am. J. Med.* **104:**33–39.
98. **Greenberg, R. N., K. Mullane, J. A. van Burik, I. Raad, M. J. Abzug, G. Anstead, R. Herbrecht, A. Langston, K. A. Marr, G. Schiller, M. Schuster, J. R. Wingard, C. E. Gonzalez, S. G. Revankar, G. Corcoran, R. J. Kryscio, and R. Hare.** 2006. Posaconazole as salvage therapy for zygomycosis. *Antimicrob. Agents Chemother.* **50:**126–133.
99. **Groll, A. H., N. Giri, V. Petraitis, R. Petraitiene, M. Candelario, J. S. Bacher, S. C. Piscitelli, and T. J. Walsh.** 2000. Comparative efficacy and distribution of lipid formulations of amphotericin B in experimental *Candida albicans* infection of the central nervous system. *J. Infect. Dis.* **182:**274–282.
100. **Groll, A. H., D. Mickiene, R. Petraitiene, V. Petraitis, C. A. Lyman, J. S. Bacher, S. C. Piscitelli, and T. J. Walsh.** 2001. Pharmacokinetic and pharmacodynamic modeling of anidulafungin (LY303366): reappraisal of its efficacy in neutropenic animal models of opportunistic mycoses using optimal plasma sampling. *Antimicrob. Agents Chemother.* **45:**2845–2855.
101. **Gubbins, P. O., G. Krishna, A. Sansone-Parsons, S. R. Penzak, L. Dong, M. Martinho, and E. J. Anaissie.** 2006. Pharmacokinetics and safety of oral posaconazole in neutropenic stem cell transplant recipients. *Antimicrob. Agents Chemother.* **50:**1993–1999.
102. **Gumbo, T., G. L. Drusano, W. Liu, L. Ma, M. R. Deziel, M. F. Drusano, and A. Louie.** 2006. Anidulafungin pharmacokinetics and microbial response in neutropenic mice with disseminated candidiasis. *Antimicrob. Agents Chemother.* **50:**3695–3700.
103. **Gumbo, T., G. L. Drusano, W. Liu, R. W. Kulawy, C. Fregeau, V. Hsu, and A. Louie.** 2007. Once-weekly micafungin therapy is as effective as daily therapy for

disseminated candidiasis in mice with persistent neutropenia. *Antimicrob. Agents Chemother.* **51:**968–974.
104. **Hardin, T. C., J. R. Graybill, R. Fetchick, R. Woestenborghs, M. G. Rinaldi, and J. G. Kuhn.** 1988. Pharmacokinetics of itraconazole following oral administration to normal volunteers. *Antimicrob. Agents Chemother.* **32:**1310–1313.
105. **Haria, M., H. M. Bryson, and K. L. Goa.** 1996. Itraconazole. A reappraisal of its pharmacological properties and therapeutic use in the management of superficial fungal infections. *Drugs* **51:**585–620.
106. **Hariprasad, S. M., W. F. Mieler, E. R. Holz, H. Gao, J. E. Kim, J. Chi, and R. A. Prince.** 2004. Determination of vitreous, aqueous, and plasma concentration of orally administered voriconazole in humans. *Arch. Ophthalmol.* **122:**42–47.
107. **Hay, R. J., Y. M. Clayton, M. K. Moore, and G. Midgely.** 1988. An evaluation of itraconazole in the management of onychomycosis. *Br. J. Dermatol.* **119:**359–366.
108. **Hebert, M. F., H. E. Smith, T. C. Marbury, S. K. Swan, W. B. Smith, R. W. Townsend, D. Buell, J. Keirns, and I. Bekersky.** 2005. Pharmacokinetics of micafungin in healthy volunteers, volunteers with moderate liver disease, and volunteers with renal dysfunction. *J. Clin. Pharmacol.* **45:**1145–1152.
109. **Heimark, L., P. Shipkova, J. Greene, H. Munayyer, T. Yarosh-Tomaine, B. DiDomenico, R. Hare, and B. N. Pramanik.** 2002. Mechanism of azole antifungal activity as determined by liquid chromatographic/mass spectrometric monitoring of ergosterol biosynthesis. *J. Mass Spectrom.* **37:**265–269.
110. **Herbrecht, R., D. W. Denning, T. F. Patterson, J. E. Bennett, R. E. Greene, J. W. Oestmann, W. V. Kern, K. A. Marr, P. Ribaud, O. Lortholary, R. Sylvester, R. H. Rubin, J. R. Wingard, P. Stark, C. Durand, D. Caillot, E. Thiel, P. H. Chandrasekar, M. R. Hodges, H. T. Schlamm, P. F. Troke, and B. de Pauw.** 2002. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N. Engl. J. Med.* **347:**408–415.
111. **Heykants, J., A. Van Peer, V. Van de Velde, P. Van Rooy, W. Meuldermans, K. Lavrijsen, R. Woestenborghs, J. Van Cutsem, and G. Cauwenbergh.** 1989. The clinical pharmacokinetics of itraconazole: an overview. *Mycoses* **32**(Suppl. 1)**:**67–87.
112. **Hitchcock, C. A., K. Dickinson, S. B. Brown, E. G. Evans, and D. J. Adams.** 1990. Interaction of azole antifungal antibiotics with cytochrome P-450-dependent 14 alpha-sterol demethylase purified from *Candida albicans*. *Biochem. J.* **266:**475–480.
113. **Hong, Y., P. J. Shaw, C. E. Nath, S. P. Yadav, K. R. Stephen, J. W. Earl, and A. J. McLachlan.** 2006. Population pharmacokinetics of liposomal amphotericin B in pediatric patients with malignant diseases. *Antimicrob. Agents Chemother.* **50:**935–942.
114. **Hope, W. W., P. A. Warn, A. Sharp, S. Howard, M. Kasai, A. Louie, T. J. Walsh, G. L. Drusano, and D. W. Denning.** 2006. Derivation of an in vivo drug exposure breakpoint for flucytosine against *Candida albicans* and Impact of the MIC, growth rate, and resistance genotype on the antifungal effect. *Antimicrob. Agents Chemother.* **50:**3680–3688.
115. **Hope, W. W., G. L. Drusano, C. B. Moore, A. Sharp, A. Louie, T. J. Walsh, D. W. Denning, and P. A. Warn.** 2007. Effect of neutropenia and treatment delay on the response to antifungal agents in experimental disseminated candidiasis. *Antimicrob. Agents Chemother.* **51:** 285–295.
116. **Hope, W. W., D. Mickiene, V. Petraitis, R. Petraitiene, A. M. Kelaher, J. E. Hughes, M. P. Cotton, J. Bacher, J. J. Keirns, D. Buell, G. Heresi, D. K. Benjamin, Jr., A. H. Groll, G. L. Drusano, and T. J. Walsh.** 2008. The pharmacokinetics and pharmacodynamics of micafungin in experimental hematogenous *Candida* meningoencephalitis: implications for echinocandin therapy in neonates. *J. Infect. Dis.* **197:**163–171.
117. **Hostetler, J. S., D. W. Denning, and D. A. Stevens.** 1992. US experience with itraconazole in *Aspergillus*, *Cryptococcus* and *Histoplasma* infections in the immunocompromised host. *Chemotherapy* **38**(Suppl. 1)**:**12–22.
118. **Hostetler, J. S., L. H. Hanson, and D. A. Stevens.** 1992. Effect of cyclodextrin on the pharmacology of antifungal oral azoles. *Antimicrob. Agents Chemother.* **36:**477–480.
119. **Hostetler, J. S., J. Heykants, K. V. Clemons, R. Woestenborghs, L. H. Hanson, and D. A. Stevens.** 1993. Discrepancies in bioassay and chromatography determinations explained by metabolism of itraconazole to hydroxyitraconazole: studies of interpatient variations in concentrations. *Antimicrob. Agents Chemother.* **37:** 2224–2227.
120. **Houang, E. T., O. Chappatte, D. Byrne, P. V. Macrae, and J. E. Thorpe.** 1990. Fluconazole levels in plasma and vaginal secretions of patients after a 150-milligram single oral dose and rate of eradication of infection in vaginal candidiasis. *Antimicrob. Agents Chemother.* **34:**909–910.
121. **Janknegt, R., S. de Marie, I. A. Bakker-Woudenberg, and D. J. Crommelin.** 1992. Liposomal and lipid formulations of amphotericin B. Clinical pharmacokinetics. *Clin. Pharmacokinet.* **23:**279–291.
122. **Johnson, L. B., and C. A. Kauffman.** 2003. Voriconazole: a new triazole antifungal agent. *Clin. Infect. Dis.* **36:**630–637.
123. **Kartsonis, N., M. J. DiNubile, K. Bartizal, P. S. Hicks, D. Ryan, and C. A. Sable.** 2002. Efficacy of caspofungin in the treatment of esophageal candidiasis resistant to fluconazole. *J. Acquir. Immune Defic. Syndr.* **31:**183–187.
124. **Katsambas, A., C. Antoniou, E. Frangouli, D. Rigopoulos, M. Vlachou, D. Michailidis, and J. Stratigos.** 1993. Itraconazole in the treatment of tinea corporis and tinea cruris. *Clin. Exp. Dermatol.* **18:**322–325.
125. **Kauffman, C. A., and P. T. Frame.** 1977. Bone marrow toxicity associated with 5-fluorocytosine therapy. *Antimicrob. Agents Chemother.* **11:**244–247.
126. **Kauffman, C. A., and P. L. Carver.** 2008. Update on echinocandin antifungals. *Semin. Respir. Crit. Care Med.* **29:**211–219.
127. **Klepser, M. E., E. J. Wolfe, R. N. Jones, C. H. Nightingale, and M. A. Pfaller.** 1997. Antifungal pharmacodynamic characteristics of fluconazole and amphotericin B tested against *Candida albicans*. *Antimicrob. Agents Chemother.* **41:**1392–1395.
128. **Kontoyiannis, D. P., R. Hachem, R. E. Lewis, G. A. Rivero, H. A. Torres, J. Thornby, R. Champlin, H. Kantarjian, G. P. Bodey, and I. I. Raad.** 2003. Efficacy and toxicity of caspofungin in combination with liposomal amphotericin B as primary or salvage treatment of invasive aspergillosis in patients with hematologic malignancies. *Cancer* **98:**292–299.
129. **Krause, D. S., A. E. Simjee, C. van Rensburg, J. Viljoen, T. J. Walsh, B. P. Goldstein, M. Wible, and T. Henkel.** 2004. A randomized, double-blind trial of anidulafungin versus fluconazole for the treatment of esophageal candidiasis. *Clin. Infect. Dis.* **39:**770–775.
130. **Krieter, P., B. Flannery, T. Musick, M. Gohdes, M. Martinho, and R. Courtney.** 2004. Disposition of posaconazole following single-dose oral administration in healthy subjects. *Antimicrob. Agents Chemother.* **48:**3543–3551.
131. **Krishna, G., A. Sansone-Parsons, M. Martinho, B. Kantesaria, and L. Pedicone.** 2007. Posaconazole plasma

concentrations in juvenile patients with invasive fungal infection. *Antimicrob. Agents Chemother.* **51:**812–818.

132. **Krishna, G., A. Moton, L. Ma, M. M. Medlock, and J. McLeod.** 2009. Pharmacokinetics and absorption of posaconazole oral suspension under various gastric conditions in healthy volunteers. *Antimicrob. Agents Chemother.* **53:** 958–966.
133. **Kullberg, B. J., J. D. Sobel, M. Ruhnke, P. G. Pappas, C. Viscoli, J. H. Rex, J. D. Cleary, E. Rubinstein, L. W. Church, J. M. Brown, H. T. Schlamm, I. T. Oborska, F. Hilton, and M. R. Hodges.** 2005. Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidaemia in non-neutropenic patients: a randomised non-inferiority trial. *Lancet* **366:**1435–1442.
134. **Kuse, E. R., P. Chetchotisakd, C. A. da Cunha, M. Ruhnke, C. Barrios, D. Raghunadharao, J. S. Sekhon, A. Freire, V. Ramasubramanian, I. Demeyer, M. Nucci, A. Leelarasamee, F. Jacobs, J. Decruyenaere, D. Pittet, A. J. Ullmann, L. Ostrosky-Zeichner, O. Lortholary, S. Koblinger, H. Diekmann-Berndt, and O. A. Cornely.** 2007. Micafungin versus liposomal amphotericin B for candidaemia and invasive candidosis: a phase III randomised double-blind trial. *Lancet* **369:**1519–1527.
135. **Lange, D., J. H. Pavao, J. Wu, and M. Klausner.** 1997. Effect of a cola beverage on the bioavailability of itraconazole in the presence of H2 blockers. *J. Clin. Pharmacol.* **37:**535–540.
136. **Lazar, J. D., and K. D. Wilner.** 1990. Drug interactions with fluconazole. *Rev. Infect. Dis.* **12**(Suppl. 3)**:**S327–S333.
137. **Lazo de la Vega, S., P. Volkow, R. A. Yeates, and G. Pfaff.** 1994. Administration of the antimycotic agents fluconazole and itraconazole to leukaemia patients: a comparative pharmacokinetic study. *Drugs Exp. Clin. Res.* **20:**69–75.
138. **Leather, H., R. M. Boyette, L. Tian, and J. R. Wingard.** 2006. Pharmacokinetic evaluation of the drug interaction between intravenous itraconazole and intravenous tacrolimus or intravenous cyclosporin A in allogeneic hematopoietic stem cell transplant recipients. *Biol. Blood Marrow Transplant.* **12:**325–334.
139. **Legendre, R., and J. Esola-Macre.** 1990. Itraconazole in the treatment of tinea capitis. *J. Am. Acad. Dermatol.* **23:**559–560.
140. **Lewis, R. E., M. E. Klepser, and M. A. Pfaller.** 2000. In vitro pharmacodynamic characteristics of flucytosine determined by time-kill methods. *Diagn. Microbiol. Infect. Dis.* **36:**101–105.
141. **Lewis, R. E., N. P. Wiederhold, and M. E. Klepser.** 2005. In vitro pharmacodynamics of amphotericin B, itraconazole, and voriconazole against *Aspergillus*, *Fusarium*, and *Scedosporium* spp. *Antimicrob. Agents Chemother.* **49:** 945–951.
142. **Li, R. K., M. A. Ciblak, N. Nordoff, L. Pasarell, D. W. Warnock, and M. R. McGinnis.** 2000. In vitro activities of voriconazole, itraconazole, and amphotericin B against *Blastomyces dermatitidis*, *Coccidioides immitis*, and *Histoplasma capsulatum*. *Antimicrob. Agents Chemother.* **44:**1734–1736.
143. **Linder, N., G. Klinger, I. Shalit, I. Levy, S. Ashkenazi, G. Haski, O. Levit, and L. Sirota.** 2003. Treatment of candidaemia in premature infants: comparison of three amphotericin B preparations. *J. Antimicrob. Chemother.* **52:**663–667.
144. **Lipp, H. P.** 2008. Antifungal agents—clinical pharmacokinetics and drug interactions. *Mycoses* **51**(Suppl. 1)**:** 7–18.
145. **Louie, A., G. L. Drusano, P. Banerjee, Q. F. Liu, W. Liu, P. Kaw, M. Shayegani, H. Taber, and M. H. Miller.** 1998. Pharmacodynamics of fluconazole in a murine model of systemic candidiasis. *Antimicrob. Agents Chemother.* **42:**1105–1109.
146. **Lutsar, I., S. Roffey, and P. Troke.** 2003. Voriconazole concentrations in the cerebrospinal fluid and brain tissue of guinea pigs and immunocompromised patients. *Clin. Infect. Dis.* **37:**728–732.
147. **Maertens, J., I. Raad, G. Petrikkos, M. Boogaerts, D. Selleslag, F. B. Petersen, C. A. Sable, N. A. Kartsonis, A. Ngai, A. Taylor, T. F. Patterson, D. W. Denning, and T. J. Walsh.** 2004. Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. *Clin. Infect. Dis.* **39:**1563–1571.
148. **Maertens, J., A. Glasmacher, R. Herbrecht, A. Thiebaut, C. Cordonnier, B. H. Segal, J. Killar, A. Taylor, N. Kartsonis, T. F. Patterson, M. Aoun, D. Caillot, and C. Sable.** 2006. Multicenter, noncomparative study of caspofungin in combination with other antifungals as salvage therapy in adults with invasive aspergillosis. *Cancer* **107:**2888–2897.
149. **Manzoni, P., I. Stolfi, L. Pugni, L. Decembrino, C. Magnani, G. Vetrano, E. Tridapalli, G. Corona, C. Giovannozzi, D. Farina, R. Arisio, F. Merletti, M. Maule, F. Mosca, R. Pedicino, M. Stronati, M. Mostert, and G. Gomirato.** 2007. A multicenter, randomized trial of prophylactic fluconazole in preterm neonates. *N. Engl. J. Med.* **356:**2483–2495.
150. **Marr, K. A., M. Boeckh, R. A. Carter, H. W. Kim, and L. Corey.** 2004. Combination antifungal therapy for invasive aspergillosis. *Clin. Infect. Dis.* **39:**797–802.
151. **McGinnis, M. R., N. Nordoff, R. K. Li, L. Pasarell, and D. W. Warnock.** 2001. *Sporothrix schenckii* sensitivity to voriconazole, itraconazole and amphotericin B. *Med. Mycol.* **39:**369–371.
152. **McNeil, M. M., S. L. Nash, R. A. Hajjeh, M. A. Phelan, L. A. Conn, B. D. Plikaytis, and D. W. Warnock.** 2001. Trends in mortality due to invasive mycotic diseases in the United States, 1980–1997. *Clin. Infect. Dis.* **33:**641–647.
153. **Meletiadis, J., J. F. Meis, J. W. Mouton, J. L. Rodriquez-Tudela, J. P. Donnelly, and P. E. Verweij.** 2002. In vitro activities of new and conventional antifungal agents against clinical *Scedosporium* isolates. *Antimicrob. Agents Chemother.* **46:**62–68.
154. **Menichetti, F., A. Del Favero, P. Martino, G. Bucaneve, A. Micozzi, C. Girmenia, G. Barbabietola, L. Pagano, P. Leoni, G. Specchia, A. Caiozzo, R. Raimondi, F. Mandelli, and the GIMEMA Infection Program.** 1999. Itraconazole oral solution as prophylaxis for fungal infections in neutropenic patients with hematologic malignancies: a randomized, placebo-controlled, double-blind, multicenter trial. *Clin. Infect. Dis.* **28:**250–255.
155. **Messer, S. A., J. T. Kirby, H. S. Sader, T. R. Fritsche, and R. N. Jones.** 2004. Initial results from a longitudinal international surveillance programme for anidulafungin (2003). *J. Antimicrob. Chemother.* **54:**1051–1056.
156. **Miller, D. J., and G. C. Mejicano.** 2001. Vertebral osteomyelitis due to *Candida* species: case report and literature review. *Clin. Infect. Dis.* **33:**523–530.
157. **Mora-Duarte, J., R. Betts, C. Rotstein, A. L. Colombo, L. Thompson-Moya, J. Smietana, R. Lupinacci, C. Sable, N. Kartsonis, and J. Perfect.** 2002. Comparison of caspofungin and amphotericin B for invasive candidiasis. *N. Engl. J. Med.* **347:**2020–2029.
158. **Moudgal, V. V., and J. D. Sobel.** 2003. Antifungal drugs in pregnancy: a review. *Expert Opin. Drug Saf.* **2:**475–483.
159. **Muhl, E., T. Martens, H. Iven, P. Rob, and H. P. Bruch.** 2000. Influence of continuous veno-venous haemodiafiltration and continuous veno-venous haemofiltration on

the pharmacokinetics of fluconazole. *Eur. J. Clin. Pharmacol.* **56:**671–678.
160. **Muhl, E.** 2005. Antimycotic drugs under continuous renal replacement therapy. *Mycoses* **48**(Suppl. 1)**:**56–60. (In German.)
161. **Munayyer, H. K., P. A. Mann, A. S. Chau, T. Yarosh-Tomaine, J. R. Greene, R. S. Hare, L. Heimark, R. E. Palermo, D. Loebenberg, and P. M. McNicholas.** 2004. Posaconazole is a potent inhibitor of sterol 14α-demethylation in yeasts and molds. *Antimicrob. Agents Chemother.* **48:**3690–3696.
162. **Nagappan, V., and S. Deresinski.** 2007. Reviews of anti-infective agents: posaconazole: a broad-spectrum triazole antifungal agent. *Clin. Infect. Dis.* **45:**1610–1617.
163. **Nakai, T., J. Uno, K. Otomo, F. Ikeda, S. Tawara, T. Goto, K. Nishimura, and M. Miyaji.** 2002. In vitro activity of FK463, a novel lipopeptide antifungal agent, against a variety of clinically important molds. *Chemotherapy* **48:**78–81.
164. **Negroni, R., A. M. Robles, A. Arechavala, and A. Taborda.** 1989. Itraconazole in human histoplasmosis. *Mycoses* **32:**123–130.
165. **Nguyen, M. H., M. L. Nguyen, V. L. Yu, D. McMahon, T. F. Keys, and M. Amidi.** 1996. *Candida* prosthetic valve endocarditis: prospective study of six cases and review of the literature. *Clin. Infect. Dis.* **22:**262–267.
166. **Nucci, M., I. Biasoli, T. Akiti, F. Silveira, C. Solza, G. Barreiros, N. Spector, A. Derossi, and W. Pulcheri.** 2000. A double-blind, randomized, placebo-controlled trial of itraconazole capsules as antifungal prophylaxis for neutropenic patients. *Clin. Infect. Dis.* **30:**300–305.
167. **Okugawa, S., Y. Ota, K. Tatsuno, K. Tsukada, S. Kishino, and K. Koike.** 2007. A case of invasive central nervous system aspergillosis treated with micafungin with monitoring of micafungin concentrations in the cerebrospinal fluid. *Scand. J. Infect. Dis.* **39:**344–346.
168. **Olkkola, K. T., J. T. Backman, and P. J. Neuvonen.** 1994. Midazolam should be avoided in patients receiving the systemic antimycotics ketoconazole or itraconazole. *Clin. Pharmacol. Ther.* **55:**481–485.
169. **Oppenheim, B. A., R. Herbrecht, and S. Kusne.** 1995. The safety and efficacy of amphotericin B colloidal dispersion in the treatment of invasive mycoses. *Clin. Infect. Dis.* **21:**1145–1153.
170. **Osser, S., A. Haglund, and L. Westrom.** 1991. Treatment of candidal vaginitis. A prospective randomized investigator-blind multicenter study comparing topically applied econazole with oral fluconazole. *Acta Obstet. Gynecol. Scand.* **70:**73–78.
171. **Ostrosky-Zeichner, L., K. A. Marr, J. H. Rex, and S. H. Cohen.** 2003. Amphotericin B: time for a new "gold standard." *Clin. Infect. Dis.* **37:**415–425.
172. **Ostrosky-Zeichner, L., D. Kontoyiannis, J. Raffalli, K. M. Mullane, J. Vazquez, E. J. Anaissie, J. Lipton, P. Jacobs, J. H. van Rensburg, J. H. Rex, W. Lau, D. Facklam, and D. N. Buell.** 2005. International, open-label, noncomparative, clinical trial of micafungin alone and in combination for treatment of newly diagnosed and refractory candidemia. *Eur. J. Clin. Microbiol. Infect. Dis.* **24:**654–661.
173. **Pai, M. P., R. S. Turpin, and K. W. Garey.** 2007. Association of fluconazole area under the concentration-time curve/MIC and dose/MIC ratios with mortality in non-neutropenic patients with candidemia. *Antimicrob. Agents Chemother.* **51:**35–39.
174. **Pappas, P. G., M. G. Threlkeld, G. D. Bedsole, K. O. Cleveland, M. S. Gelfand, and W. E. Dismukes.** 1993. Blastomycosis in immunocompromised patients. *Medicine* (Baltimore) **72:**311–325.
175. **Pappas, P. G., C. M. Rotstein, R. F. Betts, M. Nucci, D. Talwar, J. J. De Waele, J. A. Vazquez, B. F. Dupont, D. L. Horn, L. Ostrosky-Zeichner, A. C. Reboli, B. Suh, R. Digumarti, C. Wu, L. L. Kovanda, L. J. Arnold, and D. N. Buell.** 2007. Micafungin versus caspofungin for treatment of candidemia and other forms of invasive candidiasis. *Clin. Infect. Dis.* **45:**883–893.
176. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, and J. D. Sobel.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.
177. **Park, B. J., B. A. Arthington-Skaggs, R. A. Hajjeh, N. Iqbal, M. A. Ciblak, W. Lee-Yang, M. D. Hairston, M. Phelan, B. D. Plikaytis, A. N. Sofair, L. H. Harrison, S. K. Fridkin, and D. W. Warnock.** 2006. Evaluation of amphotericin B interpretive breakpoints for *Candida* bloodstream isolates by correlation with therapeutic outcome. *Antimicrob. Agents Chemother.* **50:**1287–1292.
178. **Pascual, A., T. Calandra, S. Bolay, T. Buclin, J. Bille, and O. Marchetti.** 2008. Voriconazole therapeutic drug monitoring in patients with invasive mycoses improves efficacy and safety outcomes. *Clin. Infect. Dis.* **46:**201–211.
179. **Pasqualotto, A. C., S. J. Howard, C. B. Moore, and D. W. Denning.** 2007. Flucytosine therapeutic monitoring: 15 years experience from the UK. *J. Antimicrob. Chemother.* **59:**791–793.
180. **Patel, R.** 1998. Antifungal agents. Part I. Amphotericin B preparations and flucytosine. *Mayo Clin. Proc.* **73:**1205–1225.
181. **Peng, L. W., and Y. H. Lien.** 2005. Pharmacokinetics of single, oral-dose voriconazole in peritoneal dialysis patients. *Am. J. Kidney Dis.* **45:**162–166.
182. **Perfect, J. R., W. E. Dismukes, F. Dromer, D. L. Goldman, J. R. Graybill, R. J. Hamill, T. S. Harrison, R. A. Larsen, O. Lortholary, M. H. Nguyen, P. G. Pappas, W. G. Powderly, N. Singh, J. D. Sobel, and T. C. Sorrell.** Clinical practice guidelines for the management of cryptococcal disease: 2010 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **50:**291–322.
183. **Perfect, J. R., G. M. Cox, R. K. Dodge, and W. A. Schell.** 1996. In vitro and in vivo efficacies of the azole SCH56592 against *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.* **40:**1910–1913.
184. **Perfect, J. R., K. A. Marr, T. J. Walsh, R. N. Greenberg, B. DuPont, J. de la Torre-Cisneros, G. Just-Nubling, H. T. Schlamm, I. Lutsar, A. Espinel-Ingroff, and E. Johnson.** 2003. Voriconazole treatment for less-common, emerging, or refractory fungal infections. *Clin. Infect. Dis.* **36:**1122–1131.
185. **Pfaller, M. A., L. Boyken, R. J. Hollis, J. Kroeger, S. A. Messer, S. Tendolkar, R. N. Jones, J. Turnidge, and D. J. Diekema.** 2010. Wild-type MIC distributions and epidemiological cutoff values for the echinocandins and *Candida* spp. *J. Clin. Microbiol.* **48:**52–56.
186. **Pfaller, M. A., S. A. Messer, R. J. Hollis, R. N. Jones, G. V. Doern, M. E. Brandt, and R. A. Hajjeh.** 1998. In vitro susceptibilities of *Candida* bloodstream isolates to the new triazole antifungal agents BMS-207147, Sch 56592, and voriconazole. *Antimicrob. Agents Chemother.* **42:**3242–3244.
187. **Pfaller, M. A., S. A. Messer, R. J. Hollis, and R. N. Jones.** 2001. In vitro activities of posaconazole (Sch 56592) compared with those of itraconazole and fluconazole against 3,685 clinical isolates of *Candida* spp. and *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.* **45:**2862–2864.

188. **Pfaller, M. A., S. A. Messer, R. J. Hollis, and R. N. Jones.** 2002. Antifungal activities of posaconazole, ravuconazole, and voriconazole compared to those of itraconazole and amphotericin B against 239 clinical isolates of *Aspergillus* spp. and other filamentous fungi: report from SENTRY Antimicrobial Surveillance Program, 2000. *Antimicrob. Agents Chemother.* **46:**1032–1037.
189. **Pfaller, M. A., S. A. Messer, R. J. Hollis, R. N. Jones, and D. J. Diekema.** 2002. In vitro activities of ravuconazole and voriconazole compared with those of four approved systemic antifungal agents against 6,970 clinical isolates of *Candida* spp. *Antimicrob. Agents Chemother.* **46:** 1723–1727.
190. **Pfaller, M. A., D. J. Diekema, S. A. Messer, L. Boyken, R. J. Hollis, and R. N. Jones.** 2003. In vitro activities of voriconazole, posaconazole, and four licensed systemic antifungal agents against *Candida* species infrequently isolated from blood. *J. Clin. Microbiol.* **41:**78–83.
191. **Pfaller, M. A., D. J. Diekema, S. A. Messer, R. J. Hollis, and R. N. Jones.** 2003. In vitro activities of caspofungin compared with those of fluconazole and itraconazole against 3,959 clinical isolates of *Candida* spp., including 157 fluconazole-resistant isolates. *Antimicrob. Agents Chemother.* **47:**1068–1071.
192. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2003. Caspofungin activity against clinical isolates of fluconazole-resistant *Candida*. *J. Clin. Microbiol.* **41:**5729–5731.
193. **Pfaller, M. A., and D. J. Diekema.** 2004. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J. Clin. Microbiol.* **42:**4419–4431.
194. **Pfaller, M. A., S. A. Messer, L. Boyken, R. J. Hollis, C. Rice, S. Tendolkar, and D. J. Diekema.** 2004. In vitro activities of voriconazole, posaconazole, and fluconazole against 4,169 clinical isolates of *Candida* spp. and *Cryptococcus neoformans* collected during 2001 and 2002 in the ARTEMIS global antifungal surveillance program. *Diagn. Microbiol. Infect. Dis.* **48:**201–205.
195. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, G. V. Doern, and D. J. Diekema.** 2005. Global trends in the antifungal susceptibility of *Cryptococcus neoformans* (1990 to 2004). *J. Clin. Microbiol.* **43:**2163–2167.
196. **Pfaller, M. A., D. J. Diekema, J. H. Rex, A. Espinel-Ingroff, E. M. Johnson, D. Andes, V. Chaturvedi, M. A. Ghannoum, F. C. Odds, M. G. Rinaldi, D. J. Sheehan, P. Troke, T. J. Walsh, and D. W. Warnock.** 2006. Correlation of MIC with outcome for *Candida* species tested against voriconazole: analysis and proposal for interpretive breakpoints. *J. Clin. Microbiol.* **44:** 819–826.
197. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
198. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, J. F. Meis, I. M. Gould, W. Fu, A. L. Colombo, and E. Rodriguez-Noriega.** 2007. Results from the ARTEMIS DISK Global Antifungal Surveillance study, 1997 to 2005: an 8.5-year analysis of susceptibilities of *Candida* species and other yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J. Clin. Microbiol.* **45:**1735–1745.
199. **Pfaller, M. A., D. J. Diekema, G. W. Procop, and M. G. Rinaldi.** 2007. Multicenter comparison of the VITEK 2 antifungal susceptibility test with the CLSI broth microdilution reference method for testing amphotericin B, flucytosine, and voriconazole against *Candida* spp. *J. Clin. Microbiol.* **45:**3522–3528.
200. **Pfaller, M. A., S. A. Messer, L. Boyken, C. Rice, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2007. Use of fluconazole as a surrogate marker to predict susceptibility and resistance to voriconazole among 13,338 clinical isolates of *Candida* spp. tested by Clinical and Laboratory Standards Institute-recommended broth microdilution methods. *J. Clin. Microbiol.* **45:**70–75.
201. **Phillips, P., J. Zemcov, W. Mahmood, J. S. Montaner, K. Craib, and A. M. Clarke.** 1996. Itraconazole cyclodextrin solution for fluconazole-refractory oropharyngeal candidiasis in AIDS: correlation of clinical response with in vitro susceptibility. *AIDS* **10:**1369–1376.
202. **Phillips, P., K. De Beule, G. Frechette, S. Tchamouroff, B. Vandercam, L. Weitner, A. Hoepelman, G. Stingl, and B. Clotet.** 1998. A double-blind comparison of itraconazole oral solution and fluconazole capsules for the treatment of oropharyngeal candidiasis in patients with AIDS. *Clin. Infect. Dis.* **26:**1368–1373.
203. **Pitisuttithum, P., R. Negroni, J. R. Graybill, B. Bustamante, P. Pappas, S. Chapman, R. S. Hare, and C. J. Hardalo.** 2005. Activity of posaconazole in the treatment of central nervous system fungal infections. *J. Antimicrob. Chemother.* **56:**745–755.
204. **Pitsouni, E., C. Iavazzo, and M. E. Falagas.** 2008. Itraconazole vs fluconazole for the treatment of uncomplicated acute vaginal and vulvovaginal candidiasis in nonpregnant women: a metaanalysis of randomized controlled trials. *Am. J. Obstet. Gynecol.* **198:**153–160.
205. **Poirier, J. M., F. Berlioz, F. Isnard, and G. Cheymol.** 1996. Marked intra- and inter-patient variability of itraconazole steady state plasma concentrations. *Therapie* **51:**163–167.
206. **Powderly, W. G., M. S. Saag, G. A. Cloud, P. Robinson, R. D. Meyer, J. M. Jacobson, J. R. Graybill, A. M. Sugar, V. J. McAuliffe, S. E. Follansbee, C. U. Tuazon, J. J. Stern, J. Feinberg, R. Hafner, W. E. Dismukes, and The NIAID AIDS Clinical Trials Group and Mycoses Study Group.** 1992. A controlled trial of fluconazole or amphotericin B to prevent relapse of cryptococcal meningitis in patients with the acquired immunodeficiency syndrome. *N. Engl. J. Med.* **326:**793–798.
207. **Prentice, A. G., and A. Glasmacher.** 2005. Making sense of itraconazole pharmacokinetics. *J. Antimicrob. Chemother.* **56**(Suppl. 1)**:**i17–i22.
208. **Purkins, L., N. Wood, P. Ghahramani, K. Greenhalgh, M. J. Allen, and D. Kleinermans.** 2002. Pharmacokinetics and safety of voriconazole following intravenous- to oral-dose escalation regimens. *Antimicrob. Agents Chemother.* **46:**2546–2553.
209. **Purkins, L., N. Wood, K. Greenhalgh, M. D. Eve, S. D. Oliver, and D. Nichols.** 2003. The pharmacokinetics and safety of intravenous voriconazole—a novel wide-spectrum antifungal agent. *Br. J. Clin. Pharmacol.* **56**(Suppl. 1)**:**2–9.
210. **Purkins, L., N. Wood, D. Kleinermans, K. Greenhalgh, and D. Nichols.** 2003. Effect of food on the pharmacokinetics of multiple-dose oral voriconazole. *Br. J. Clin. Pharmacol.* **56**(Suppl. 1)**:**17–23.
211. **Pursley, T. J., I. K. Blomquist, J. Abraham, H. F. Andersen, and J. A. Bartley.** 1996. Fluconazole-induced congenital anomalies in three infants. *Clin. Infect. Dis.* **22:**336–340.
212. **Raad, I. I., R. Y. Hachem, R. Herbrecht, J. R. Graybill, R. Hare, G. Corcoran, and D. P. Kontoyiannis.** 2006. Posaconazole as salvage treatment for invasive fusariosis in patients with underlying hematologic malignancy and other conditions. *Clin. Infect. Dis.* **42:**1398–1403.
213. **Reboli, A. C., C. Rotstein, P. G. Pappas, S. W. Chapman, D. H. Kett, D. Kumar, R. Betts, M. Wible, B. P.**

Goldstein, J. Schranz, D. S. Krause, and T. J. Walsh. 2007. Anidulafungin versus fluconazole for invasive candidiasis. *N. Engl. J. Med.* **356:**2472–2482.
214. **Restrepo, A., J. Robledo, I. Gomez, A. M. Tabares, and R. Gutierrez.** 1986. Itraconazole therapy in lymphangitic and cutaneous sporotrichosis. *Arch. Dermatol.* **122:** 413–417.
215. **Restrepo, A.** 1994. Treatment of tropical mycoses. *J. Am. Acad. Dermatol.* **31:**S91–S102.
216. **Restrepo, A., A. Tobon, B. Clark, D. R. Graham, G. Corcoran, R. W. Bradsher, M. Goldman, G. Pankey, T. Moore, R. Negroni, and J. R. Graybill.** 2007. Salvage treatment of histoplasmosis with posaconazole. *J. Infect.* **54:**319–327.
217. **Rex, J. H., J. E. Bennett, A. M. Sugar, P. G. Pappas, C. M. van der Horst, J. E. Edwards, R. G. Washburn, W. M. Scheld, A. W. Karchmer, A. P. Dine, M. J. Levenstein, and C. D. Webb for the Candidemia Study Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group.** 1994. A randomized trial comparing fluconazole with amphotericin B for the treatment of candidemia in patients without neutropenia. *N. Engl. J. Med.* **331:**1325–1330.
218. **Rex, J. H., M. A. Pfaller, J. N. Galgiani, M. S. Bartlett, A. Espinel-Ingroff, M. A. Ghannoum, M. Lancaster, F. C. Odds, M. G. Rinaldi, T. J. Walsh, and A. L. Barry.** 1997. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and *Candida* infections. Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards. *Clin. Infect. Dis.* **24:**235–247.
219. **Rodriguez-Tudela, J. L., B. Almirante, D. Rodriguez-Pardo, F. Laguna, J. P. Donnelly, J. W. Mouton, A. Pahissa, and M. Cuenca-Estrella.** 2007. Correlation of the MIC and dose/MIC ratio of fluconazole to the therapeutic response of patients with mucosal candidiasis and candidemia. *Antimicrob. Agents Chemother.* **51:**3599–3604.
220. **Saag, M. S., and W. E. Dismukes.** 1988. Azole antifungal agents: emphasis on new triazoles. *Antimicrob. Agents Chemother.* **32:**1–8.
221. **Saag, M. S., W. G. Powderly, G. A. Cloud, P. Robinson, M. H. Grieco, P. K. Sharkey, S. E. Thompson, A. M. Sugar, C. U. Tuazon, J. F. Fisher, N. Hyslop, J. M. Jacobson, R. Hafner, W. E. Dismukes, and the NIAID Mycoses Study Group and the AIDS Clinical Trials Group.** 1992. Comparison of amphotericin B with fluconazole in the treatment of acute AIDS-associated cryptococcal meningitis. *N. Engl. J. Med.* **326:**83–89.
222. **Saag, M. S., G. A. Cloud, J. R. Graybill, J. D. Sobel, C. U. Tuazon, P. C. Johnson, W. J. Fessel, B. L. Moskovitz, B. Wiesinger, D. Cosmatos, L. Riser, C. Thomas, R. Hafner, and W. E. Dismukes.** 1999. A comparison of itraconazole versus fluconazole as maintenance therapy for AIDS-associated cryptococcal meningitis. National Institute of Allergy and Infectious Diseases Mycoses Study Group. *Clin. Infect. Dis.* **28:**291–296.
223. **Saag, M. S., W. J. Fessel, C. A. Kaufman, K. W. Merrill, D. J. Ward, B. L. Moskovitz, C. Thomas, N. Oleka, J. A. Guarnieri, J. Lee, L. Brenner-Gati, and M. Klausner.** 1999. Treatment of fluconazole-refractory oropharyngeal candidiasis with itraconazole oral solution in HIV-positive patients. *AIDS Res. Hum. Retrovir.* **15:**1413–1417.
224. **Sabatelli, F., R. Patel, P. A. Mann, C. A. Mendrick, C. C. Norris, R. Hare, D. Loebenberg, T. A. Black, and P. M. McNicholas.** 2006. In vitro activities of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. *Antimicrob. Agents Chemother.* **50:** 2009–2015.
225. **Sanati, H., P. Belanger, R. Fratti, and M. Ghannoum.** 1997. A new triazole, voriconazole (UK-109,496), blocks sterol biosynthesis in *Candida albicans* and *Candida krusei*. *Antimicrob. Agents Chemother.* **41:**2492–2496.
226. **Sandhu, P., W. Lee, X. Xu, B. F. Leake, M. Yamazaki, J. A. Stone, J. H. Lin, P. G. Pearson, and R. B. Kim.** 2005. Hepatic uptake of the novel antifungal agent caspofungin. *Drug Metab Dispos.* **33:**676–682.
227. **Savani, D. V., J. R. Perfect, L. M. Cobo, and D. T. Durack.** 1987. Penetration of new azole compounds into the eye and efficacy in experimental *Candida* endophthalmitis. *Antimicrob. Agents Chemother.* **31:**6–10.
228. **Schmitt-Hoffmann, A., B. Roos, J. Spickermann, M. Heep, E. Peterfai, D. J. Edwards, and K. Stoeckel.** 2009. Effect of mild and moderate liver disease on the pharmacokinetics of isavuconazole after intravenous and oral administration of a single dose of the prodrug BAL8557. *Antimicrob. Agents Chemother.* **53:**4885–4890.
229. **Schonebeck, J., A. Polak, M. Fernex, and H. J. Scholer.** 1973. Pharmacokinetic studies on the oral antimycotic agent 5-fluorocytosine in individuals with normal and impaired kidney function. *Chemotherapy* **18:**321–336.
230. **Schuster, M. G., J. E. Edwards, Jr., J. D. Sobel, R. O. Darouiche, A. W. Karchmer, S. Hadley, G. Slotman, H. Panzer, P. Biswas, and J. H. Rex.** 2008. Empirical fluconazole versus placebo for intensive care unit patients: a randomized trial. *Ann. Intern. Med.* **149:**83–90.
231. **Shiba, K., A. Saito, and T. Miyahara.** 1990. Safety and pharmacokinetics of single oral and intravenous doses of fluconazole in healthy subjects. *Clin. Ther.* **12:**206–215.
232. **Shorr, A. F., K. Chung, W. L. Jackson, P. E. Waterman, and M. H. Kollef.** 2005. Fluconazole prophylaxis in critically ill surgical patients: a meta-analysis. *Crit. Care Med.* **33:**1928–1935; quiz, 1936.
233. **Skiest, D. J., J. A. Vazquez, G. M. Anstead, J. R. Graybill, J. Reynes, D. Ward, R. Hare, N. Boparai, and R. Isaacs.** 2007. Posaconazole for the treatment of azole-refractory oropharyngeal and esophageal candidiasis in subjects with HIV infection. *Clin. Infect. Dis.* **44:**607–614.
234. **Slavin, M. A., B. Osborne, R. Adams, M. J. Levenstein, H. G. Schoch, A. R. Feldman, J. D. Meyers, and R. A. Bowden.** 1995. Efficacy and safety of fluconazole prophylaxis for fungal infections after marrow transplantation—a prospective, randomized, double-blind study. *J. Infect. Dis.* **171:**1545–1552.
235. **Smith, D. E., J. Midgley, M. Allan, G. M. Connolly, and B. G. Gazzard.** 1991. Itraconazole versus ketaconazole in the treatment of oral and oesophageal candidosis in patients infected with HIV. *AIDS* **5:**1367–1371.
236. **Smith, J., N. Safdar, V. Knasinski, W. Simmons, S. M. Bhavnani, P. G. Ambrose, and D. Andes.** 2006. Voriconazole therapeutic drug monitoring. *Antimicrob. Agents Chemother.* **50:**1570–1572.
237. **Smith, J., and D. Andes.** 2008. Therapeutic drug monitoring of antifungals: pharmacokinetic and pharmacodynamic considerations. *Ther. Drug Monit.* **30:** 167–172.
238. **Sobel, J. D., D. Brooker, G. E. Stein, J. L. Thomason, D. P. Wermeling, B. Bradley, and L. Weinstein.** 1995. Single oral dose fluconazole compared with conventional clotrimazole topical therapy of *Candida* vaginitis. Fluconazole Vaginitis Study Group. *Am. J. Obstet. Gynecol.* **172:**1263–1268.
239. **Sobel, J. D., P. S. Kapernick, M. Zervos, B. D. Reed, T. Hooton, D. Soper, P. Nyirjesy, M. W. Heine, J. Willems, H. Panzer, and H. Wittes.** 2001. Treatment of complicated *Candida* vaginitis: comparison of single

and sequential doses of fluconazole. *Am. J. Obstet. Gynecol.* **185:**363–369.
240. **Spellberg, B., T. J. Walsh, D. P. Kontoyiannis, J. Edwards, Jr., and A. S. Ibrahim.** 2009. Recent advances in the management of mucormycosis: from bench to bedside. *Clin. Infect. Dis.* **48:**1743–1751.
241. **Sponsel, W. E., J. R. Graybill, H. L. Nevarez, and D. Dang.** 2002. Ocular and systemic posaconazole (SCH-56592) treatment of invasive *Fusarium solani* keratitis and endophthalmitis. *Br. J. Ophthalmol.* **86:**829–830.
242. **Stamm, A. M., R. B. Diasio, W. E. Dismukes, S. Shadomy, G. A. Cloud, C. A. Bowles, G. H. Karam, and A. Espinel-Ingroff.** 1987. Toxicity of amphotericin B plus flucytosine in 194 patients with cryptococcal meningitis. *Am. J .Med.* **83:**236–242.
243. **Stevens, D. A., and J. Y. Lee.** 1997. Analysis of compassionate use itraconazole therapy for invasive aspergillosis by the NIAID Mycoses Study Group criteria. *Arch. Intern. Med.* **157:**1857–1862.
244. **Stevens, D. A.** 1999. Itraconazole in cyclodextrin solution. *Pharmacotherapy* **19:**603–611.
245. **Stevens, D. A., A. Rendon, V. Gaona-Flores, A. Catanzaro, G. M. Anstead, L. Pedicone, and J. R. Graybill.** 2007. Posaconazole therapy for chronic refractory coccidioidomycosis. *Chest* **132:**952–958.
246. **Sugar, A. M., and X. P. Liu.** 1996. In vitro and in vivo activities of SCH 56592 against *Blastomyces dermatitidis*. *Antimicrob. Agents Chemother.* **40:**1314–1316.
247. **Sun, Q. N., A. W. Fothergill, D. I. McCarthy, M. G. Rinaldi, and J. R. Graybill.** 2002. In vitro activities of posaconazole, itraconazole, voriconazole, amphotericin B, and fluconazole against 37 clinical isolates of zygomycetes. *Antimicrob. Agents Chemother.* **46:**1581–1582.
248. **Svejgaard, E., C. Avnstorp, B. Wanscher, J. Nilsson, and A. Heremans.** 1998. Efficacy and safety of short-term itraconazole in tinea pedis: a double-blind, randomized, placebo-controlled trial. *Dermatology* **197:**368–372.
249. **Takakura, S., N. Fujihara, T. Saito, T. Kudo, Y. Iinuma, and S. Ichiyama.** 2004. Clinical factors associated with fluconazole resistance and short-term survival in patients with *Candida* bloodstream infection. *Eur. J. Clin. Microbiol. Infect. Dis.* **23:**380–388.
250. **Tassel, D., and M. A. Madoff.** 1968. Treatment of *Candida* sepsis and *Cryptococcus* meningitis with 5-fluorocytosine. A new antifungal agent. *JAMA* **206:**830–832.
251. **Thaler, F., B. Bernard, M. Tod, C. P. Jedynak, O. Petitjean, P. Derome, and P. Loirat.** 1995. Fluconazole penetration in cerebral parenchyma in humans at steady state. *Antimicrob. Agents Chemother.* **39:**1154–1156.
252. **Theuretzbacher, U., F. Ihle, and H. Derendorf.** 2006. Pharmacokinetic/pharmacodynamic profile of voriconazole. *Clin. Pharmacokinet.* **45:**649–663.
253. **Thiel, M. A., A. S. Zinkernagel, J. Burhenne, C. Kaufmann, and W. E. Haefeli.** 2007. Voriconazole concentration in human aqueous humor and plasma during topical or combined topical and systemic administration for fungal keratitis. *Antimicrob. Agents Chemother.* **51:** 239–244.
254. **Thompson, G. R., III, J. Cadena, and T. F. Patterson.** 2009. Overview of antifungal agents. *Clin. Chest Med.* **30:**203–215.
255. **Thorpe, J. E., N. Baker, and M. Bromet-Petit.** 1990. Effect of oral antacid administration on the pharmacokinetics of oral fluconazole. *Antimicrob. Agents Chemother.* **34:**2032–2033.
256. **Timonen, H.** 1992. Shorter treatment for vaginal candidosis: comparison between single-dose oral fluconazole and three-day treatment with local miconazole. *Mycoses* **35:**317–320.
257. **Tobin, J. M., P. Loo, and S. E. Granger.** 1992. Treatment of vaginal candidosis: a comparative study of the efficacy and acceptability of itraconazole and clotrimazole. *Genitourin. Med.* **68:**36–38.
258. **Tollemar, J., S. Andersson, O. Ringden, and G. Tyden.** 1992. A retrospective clinical comparison between antifungal treatment with liposomal amphotericin B (AmBisome) and conventional amphotericin B in transplant recipients. *Mycoses* **35:**215–220.
259. **Trifilio, S., R. Ortiz, G. Pennick, A. Verma, J. Pi, V. Stosor, T. Zembower, and J. Mehta.** 2005. Voriconazole therapeutic drug monitoring in allogeneic hematopoietic stem cell transplant recipients. *Bone Marrow Transplant.* **35:**509–513.
260. **Tucker, R. M., D. W. Denning, E. G. Arathoon, M. G. Rinaldi, and D. A. Stevens.** 1990. Itraconazole therapy for nonmeningeal coccidioidomycosis: clinical and laboratory observations. *J. Am. Acad. Dermatol.* **23:**593–601.
261. **Tucker, R. M., D. W. Denning, L. H. Hanson, M. G. Rinaldi, J. R. Graybill, P. K. Sharkey, D. Pappagianis, and D. A. Stevens.** 1992. Interaction of azoles with rifampin, phenytoin, and carbamazepine: in vitro and clinical observations. *Clin. Infect. Dis.* **14:**165–174.
262. **Turnidge, J. D., S. Gudmundsson, B. Vogelman, and W. A. Craig.** 1994. The postantibiotic effect of antifungal agents against common pathogenic yeasts. *J. Antimicrob. Chemother.* **34:**83–92.
263. **Ullmann, A. J., O. A. Cornely, A. Burchardt, R. Hachem, D. P. Kontoyiannis, K. Topelt, R. Courtney, D. Wexler, G. Krishna, M. Martinho, G. Corcoran, and I. Raad.** 2006. Pharmacokinetics, safety, and efficacy of posaconazole in patients with persistent febrile neutropenia or refractory invasive fungal infection. *Antimicrob. Agents Chemother.* **50:**658–666.
264. **Ullmann, A. J., J. H. Lipton, D. H. Vesole, P. Chandrasekar, A. Langston, S. R. Tarantolo, H. Greinix, W. Morais de Azevedo, V. Reddy, N. Boparai, L. Pedicone, H. Patino, and S. Durrant.** 2007. Posaconazole or fluconazole for prophylaxis in severe graft-versus-host disease. *N. Engl. J. Med.* **356:**335–347.
265. **Valtonen, M., E. Tiula, and P. J. Neuvonen.** 1997. Effect of continuous venovenous haemofiltration and haemodiafiltration on the elimination of fluconazole in patients with acute renal failure. *J. Antimicrob. Chemother.* **40:** 695–700.
266. **van Burik, J. A., V. Ratanatharathorn, D. E. Stepan, C. B. Miller, J. H. Lipton, D. H. Vesole, N. Bunin, D. A. Wall, J. W. Hiemenz, Y. Satoi, J. M. Lee, and T. J. Walsh.** 2004. Micafungin versus fluconazole for prophylaxis against invasive fungal infections during neutropenia in patients undergoing hematopoietic stem cell transplantation. *Clin. Infect. Dis.* **39:**1407–1416.
267. **Vanden Bossche, H.** 1985. Biochemical targets for antifungal azole derivatives: hypothesis on the mode of action. *Curr. Top. Med. Mycol.* **1:**313–351.
268. **van Heusden, A. M., H. M. Merkus, R. Euser, and A. Verhoeff.** 1994. A randomized, comparative study of a single oral dose of fluconazole versus a single topical dose of clotrimazole in the treatment of vaginal candidosis among general practitioners and gynaecologists. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **55:**123–127.
269. **Van Peer, A., R. Woestenborghs, J. Heykants, R. Gasparini, and G. Gauwenbergh.** 1989. The effects of food and dose on the oral systemic availability of itraconazole in healthy subjects. *Eur. J. Clin. Pharmacol.* **36:** 423–426.
270. **van't Wout, J. W., I. Novakova, C. A. Verhagen, W. E. Fibbe, B. E. de Pauw, and J. W. van der Meer.** 1991. The

efficacy of itraconazole against systemic fungal infections in neutropenic patients: a randomised comparative study with amphotericin B. *J. Infect.* **22:**45–52.

271. **Vardakas, K. Z., A. Michalopoulos, and M. E. Falagas.** 2005. Fluconazole versus itraconazole for antifungal prophylaxis in neutropenic patients with haematological malignancies: a meta-analysis of randomised-controlled trials. *Br. J. Haematol.* **131:**22–28.
272. **Vazquez, J. A., D. J. Skiest, L. Nieto, R. Northland, I. Sanne, J. Gogate, W. Greaves, and R. Isaacs.** 2006. A multicenter randomized trial evaluating posaconazole versus fluconazole for the treatment of oropharyngeal candidiasis in subjects with HIV/AIDS. *Clin. Infect. Dis.* **42:**1179–1186.
273. **Vazquez, J. A., and J. D. Sobel.** 2006. Anidulafungin: a novel echinocandin. *Clin. Infect. Dis.* **43:**215–222.
274. **Vazquez, J. A., D. J. Skiest, H. Tissot-Dupont, J. L. Lennox, N. Boparai, and R. Isaacs.** 2007. Safety and efficacy of posaconazole in the long-term treatment of azole-refractory oropharyngeal and esophageal candidiasis in patients with HIV infection. *HIV Clin. Trials* **8:** 86–97.
275. **Vermes, A., H. J. Guchelaar, and J. Dankert.** 2000. Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. *J. Antimicrob. Chemother.* **46:**171–179.
276. **Villanueva, A., E. G. Arathoon, E. Gotuzzo, R. S. Berman, M. J. DiNubile, and C. A. Sable.** 2001. A randomized double-blind study of caspofungin versus amphotericin for the treatment of candidal esophagitis. *Clin. Infect. Dis.* **33:**1529–1535.
277. **Villanueva, A., E. Gotuzzo, E. G. Arathoon, L. M. Noriega, N. A. Kartsonis, R. J. Lupinacci, J. M. Smietana, M. J. DiNubile, and C. A. Sable.** 2002. A randomized double-blind study of caspofungin versus fluconazole for the treatment of esophageal candidiasis. *Am. J. Med.* **113:**294–299.
278. **Vogelman, B., S. Gudmundsson, J. Turnidge, J. Leggett, and W. A. Craig.** 1988. In vivo postantibiotic effect in a thigh infection in neutropenic mice. *J. Infect. Dis.* **157:**287–298.
279. **Wagner, C., W. Graninger, E. Presterl, and C. Joukhadar.** 2006. The echinocandins: comparison of their pharmacokinetics, pharmacodynamics and clinical applications. *Pharmacology* **78:**161–177.
280. **Waldorf, A. R., and A. Polak.** 1983. Mechanisms of action of 5-fluorocytosine. *Antimicrob. Agents Chemother.* **23:**79–85.
281. **Walsh, T. J., G. Foulds, and P. A. Pizzo.** 1989. Pharmacokinetics and tissue penetration of fluconazole in rabbits. *Antimicrob. Agents Chemother.* **33:**467–469.
282. **Walsh, T. J., J. W. Hiemenz, N. L. Seibel, J. R. Perfect, G. Horwith, L. Lee, J. L. Silber, M. J. DiNubile, A. Reboli, E. Bow, J. Lister, and E. J. Anaissie.** 1998. Amphotericin B lipid complex for invasive fungal infections: analysis of safety and efficacy in 556 cases. *Clin. Infect. Dis.* **26:**1383–1396.
283. **Walsh, T. J., M. O. Karlsson, T. Driscoll, A. G. Arguedas, P. Adamson, X. Saez-Llorens, A. J. Vora, A. C. Arrieta, J. Blumer, I. Lutsar, P. Milligan, and N. Wood.** 2004. Pharmacokinetics and safety of intravenous voriconazole in children after single- or multiple-dose administration. *Antimicrob. Agents Chemother.* **48:**2166–2172.
284. **Walsh, T. J., H. Teppler, G. R. Donowitz, J. A. Maertens, L. R. Baden, A. Dmoszynska, O. A. Cornely, M. R. Bourque, R. J. Lupinacci, C. A. Sable, and B. E. dePauw.** 2004. Caspofungin versus liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia. *N. Engl. J. Med.* **351:** 1391–1402.
285. **Walsh, T. J., I. Raad, T. F. Patterson, P. Chandrasekar, G. R. Donowitz, R. Graybill, R. E. Greene, R. Hachem, S. Hadley, R. Herbrecht, A. Langston, A. Louie, P. Ribaud, B. H. Segal, D. A. Stevens, J. A. van Burik, C. S. White, G. Corcoran, J. Gogate, G. Krishna, L. Pedicone, C. Hardalo, and J. R. Perfect.** 2007. Treatment of invasive aspergillosis with posaconazole in patients who are refractory to or intolerant of conventional therapy: an externally controlled trial. *Clin. Infect. Dis.* **44:**2–12.
286. **Walsh, T. J., E. J. Anaissie, D. W. Denning, R. Herbrecht, D. P. Kontoyiannis, K. A. Marr, V. A. Morrison, B. H. Segal, W. J. Steinbach, D. A. Stevens, J. A. van Burik, J. R. Wingard, and T. F. Patterson.** 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. *Clin. Infect. Dis.* **46:**327–360.
287. **Walsoe, I., M. Stangerup, and E. Svejgaard.** 1990. Itraconazole in onychomycosis. Open and double-blind studies. *Acta Derm. Venereol.* **70:**137–140.
288. **Warn, P. A., J. Morrissey, C. B. Moore, and D. W. Denning.** 2000. In vivo activity of amphotericin B lipid complex in immunocompromised mice against fluconazole-resistant or fluconazole-susceptible *Candida tropicalis*. *Antimicrob. Agents Chemother.* **44:**2664–2671.
289. **Warn, P. A., A. Sharp, A. Parmar, J. Majithiya, D. W. Denning, and W. W. Hope.** 2009. Pharmacokinetics and pharmacodynamics of a novel triazole, isavuconazole: mathematical modeling, importance of tissue concentrations, and impact of immune status on antifungal effect. *Antimicrob. Agents Chemother.* **53:**3453–3461.
290. **Warnock, D. W., A. Turner, and J. Burke.** 1988. Comparison of high performance liquid chromatographic and microbiological methods for determination of itraconazole. *J. Antimicrob. Chemother.* **21:**93–100.
291. **Wheat, J., R. Hafner, A. H. Korzun, M. T. Limjoco, P. Spencer, R. A. Larsen, F. M. Hecht, and W. Powderly.** 1995. Itraconazole treatment of disseminated histoplasmosis in patients with the acquired immunodeficiency syndrome. AIDS Clinical Trial Group. *Am. J. Med.* **98:** 336–342.
292. **Wheat, L. J., A. G. Freifeld, M. B. Kleiman, J. W. Baddley, D. S. McKinsey, J. E. Loyd, and C. A. Kauffman.** 2007. Clinical practice guidelines for the management of patients with histoplasmosis: 2007 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **45:** 807–825.
293. **Wiederhold, N. P., D. P. Kontoyiannis, J. Chi, R. A. Prince, V. H. Tam, and R. E. Lewis.** 2004. Pharmacodynamics of caspofungin in a murine model of invasive pulmonary aspergillosis: evidence of concentration-dependent activity. *J. Infect. Dis.* **190:**1464–1471.
294. **Wiederhold, N. P.** 2009. Paradoxical echinocandin activity: a limited in vitro phenomenon? *Med. Mycol.* **47** (Suppl. 1)**:**S369–S375.
295. **Wilcox, C. M., R. O. Darouiche, L. Laine, B. L. Moskovitz, I. Mallegol, and J. Wu.** 1997. A randomized, double-blind comparison of itraconazole oral solution and fluconazole tablets in the treatment of esophageal candidiasis. *J. Infect. Dis.* **176:**227–232.
296. **Willems, L., R. van der Geest, and K. de Beule.** 2001. Itraconazole oral solution and intravenous formulations: a review of pharmacokinetics and pharmacodynamics. *J. Clin. Pharm. Ther.* **26:**159–169.

297. **Winston, D. J., and R. W. Busuttil.** 2002. Randomized controlled trial of oral itraconazole solution versus intravenous/oral fluconazole for prevention of fungal infections in liver transplant recipients. *Transplantation* **74:**688–695.
298. **Yano, T., Y. Itoh, E. Kawamura, A. Maeda, N. Egashira, M. Nishida, H. Kurose, and R. Oishi.** 2009. Amphotericin B-induced renal tubular cell injury is mediated by Na^+ influx through ion-permeable pores and subsequent activation of mitogen-activated protein kinases and elevation of intracellular Ca^{2+} concentration. *Antimicrob. Agents Chemother.* **53:**1420–1426.
299. **Yeh, J., S. C. Soo, C. Summerton, and C. Richardson.** 1990. Potentiation of action of warfarin by itraconazole. *BMJ* **301:**669.
300. **Zimmermann, T., R. A. Yeates, H. Laufen, G. Pfaff, and A. Wildfeuer.** 1994. Influence of concomitant food intake on the oral absorption of two triazole antifungal agents, itraconazole and fluconazole. *Eur. J. Clin. Pharmacol.* **46:**147–150.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy
©2012 ASM Press, Washington, DC
10.1128/9781555817176.ch23

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The Impact of Antifungal Drug Resistance in the Clinic

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Over the past two decades, virtually every publication on the topic of antifungal resistance in *Candida* species has begun with descriptions of (i) the increasing impact of antifungal resistance in the management of invasive candidiasis, (ii) reiteration of a globally shifting epidemiology in *Candida* infections towards resistant non-*Candida albicans Candida* species, and (iii) a plea for improved diagnostic approaches and novel antifungal agents to combat the rising tide of antifungal resistance. While these statements have some truth, they somewhat distort and oversimplify the clinical reality of antifungal resistance encountered by clinicians who are caring for patients with invasive candidiasis. In this chapter, we critically evaluate some common assumptions regarding antifungal resistance and highlight key clinical problems that arise when managing patients with invasive infections caused by antifungal-resistant *Candida* species. Where possible, we provide practical recommendations for managing patients with suspected or documented infections due to resistant *Candida* species.

WHAT IMPACT DOES ANTIFUNGAL RESISTANCE HAVE ON THE MORTALITY OF INVASIVE CANDIDIASIS?

Resistance and Attributable Mortality

Candida species are the most common cause of invasive fungal infections in humans, producing syndromes that range from self-limiting mucocutaneous infections to hyperacute invasive infections that can disseminate to any organ (92, 93). In hospitals, *Candida* species are the third to fourth most commonly isolated bloodstream pathogens, accounting for 8 to 15% of all bloodstream infections (48, 93, 114, 121). The most common risk factors for invasive candidiasis include major surgery (especially involving the abdomen), immunosuppression, and supportive care measures used in the critically ill patient such as broad-spectrum antimicrobials, total parenteral nutrition, renal replacement therapies, and central venous catheters (48, 70, 71, 122, 123). Extremes of age and underlying comorbidities such as diabetes mellitus and chronic malnutrition can further enhance susceptibility to invasive candidiasis (48).

Many clinical risk factors that predispose patients to invasive candidiasis overlap with risk factors for acquiring multidrug-resistant (MDR) bacterial and fungal pathogens (7). As a result, *Candida* bloodstream infections, particularly infections caused by antifungal-resistant *Candida* species such as *C. glabrata* and *C. krusei*, are primarily encountered in patients with poor prognosis for survival due to the severity of their underlying disease (7, 33). In fact, many clinicians consider candidemia to be more of an indicator of worsening clinical status or an end-stage event associated with incurable underlying illness rather than an independent complication causing patient death (15). While these end-stage patients are typically excluded from prospective randomized clinical trials, they are rarely excluded from single-center retrospective studies examining outcomes of infection caused by resistant *Candida* species (7, 54). As a result, antifungal resistance is often portrayed in retrospective studies as an important contributor to patient death.

Although the attributable mortality rates of 25 to 47% for candidemia are widely reported in the medical literature (43, 56, 118, 119, 121), some carefully performed case-matched cohort studies of candidemic patients have estimated lower attributable mortality rates, in the range of 10 to 15% (16, 33). Therefore, thousands of candidemic patients may be required in any case-cohort study to generate sufficient statistical power to identify antifungal resistance in *Candida* species as an independent predictor of patient mortality. In fact, prospective case-cohort trials that have focused on predictors of poor outcome in patients with invasive candidiasis have consistently failed to identify antifungal resistance as an independent risk factor for patient death (Table 1) (48, 62, 83, 113, 116). These studies have shown consistently that underlying disease severity (i.e., APACHE II score), extremes of age, severe immunosuppression, and possibly lack of timely catheter removal are the key variables associated with death of patients with canididemia 30 to 45 days after a positive index blood culture (Table 1).

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TABLE 1 Predictors of poor outcome in recent prospective multicenter observational cohort studies of invasive candidiasis

Investigator(s) (year)	Study period	Patient population	Attributable and crude mortality	Independent variables influencing patient outcome in multivariate analysis	Comments
Viscoli et al., 1999 (116)	1992–1994	Cancer hospitals in Europe *n* = 249 patients	Attributable, 8% Crude, 39%	Solid-tumor patients Age Stage of disease Hematologic malignancy Age Stage of disease Last chemotherapy Lack of antifungal prophylaxis Shock	30 tertiary-care university medical centers in Europe No evidence of increased mortality rate associated with fluconazole-resistant *Candida* species Lower mortality rates were observed with *C. parapsilosis*
Pappas et al., 2003 (83)	1995–1997	Heterogeneous; medical centers participating in phase III clinical trial; *n* = 1,447 adults; *n* = 144 children	Attributable, 12% Crude, 60%	Adults APACHE II, >18 Underlying cancer Urinary catheter Male sex Infection by species other than *C. parapsilosis* Receipt of corticosteroids Arterial catheter Children Neutropenia Intubation	No differences were observed among patients treated with amphotericin B versus fluconazole, or in patients who received fluconazole for *C. glabrata* versus other antifungal therapies
Leroy et al., 2009 (62)	2005–2006	ICUs in France; *n* = 271	Attributable, not reported Crude, 52%	Type 1 diabetes Immunosuppression Mechanical ventilation Body temp, >38.2°C	No difference in mortality rates between patients with infections due to fluconazole-susceptible versus susceptible-dose-dependent or resistant species
Slavin et al., 2010 (113)	2001–2004	Cancer patients in Australia; *n* = 138 hematologic malignancy; *n* = 150 solid tumor	Attributable Hematologic, 13.2%; Solid tumor, 12.1% Crude mortality Hematologic, 40% Solid tumor, 45%	Removal of central venous catheter	Trend towards delay in clearance of blood cultures, poorer clinical course, and reduced survival observed in patients with fluconazole-resistant candidemia
Horn et al., 2009 (48)	2003–2006	Heterogeneous; *n* = 1,070 adult patients	Not reported	APACHE II >20 Persistent neutropenia Age, >70 yrs Asian-Indian ethnicity	Pooled post hoc analysis of patients enrolled in two prospective randomized candidemia trials Species isolated at baseline was not associated with mortality

Furthermore, none of these studies demonstrated clear evidence of increased mortality associated with fluconazole-resistant *Candida* species, including for patients who were initially treated inappropriately with fluconazole (48, 54). With the declining number of autopsies performed in most institutions, it has become increasingly difficult to assess the true attributable mortality of invasive candidiasis and its corresponding impact of antifungal resistance (22, 112).

Resistance and Early Appropriate Therapy

Early appropriate therapy has been shown to be an important predictor of outcome for patients with microbiologi-

cally confirmed nosocomial infections, including *Candida* bloodstream infections (49, 64). In a frequently cited single-center retrospective study, Morrell and colleagues reported that the risk of all-cause mortality in the hospital for candidemic patients increased from 11.1 to 33.1% if antifungal treatment was not started within 12 h of a positive blood culture (68). However, their analysis did not demonstrate a significant difference in the rate of persistent or recurrent fungemia between survivors versus nonsurvivors, and it demonstrated virtually no difference in the rate of "inappropriate" treatment was reported for patients infected with *C. albicans* (96.4%) versus more antifungal-resistant *C. glabrata* (95%) and *C. krusei* (100%) (68).

A similar multicenter case-cohort trial performed by Garey and colleagues found that delays in the initiation of fluconazole therapy by more than 24 h from the time of onset of symptoms were associated with progressively higher all-cause mortality, although infection with fluconazole-resistant *Candida* species did not emerge as an independent predictor of death in the multivariate analysis (40). In a follow-up analysis of their database that focused on the relationship of fluconazole dosing and patient mortality, the investigators found significantly higher mortality rates in patients with estimated fluconazole dose/MIC ratios of <15 when the time to initiation of fluconazole therapy was controlled in their analysis (80). Patients with *C. glabrata* infection ($n = 11$) were more likely to have low weight-normalized fluconazole daily dose/MIC ratios than were patients with *C. albicans* (1.9 versus 15.4, respectively), as well as higher crude mortality rates (36.4% versus 16.3%) (80). Yet among the 77 patients included in the analysis, there were only 2 patients infected with fluconazole susceptible-dose-dependent isolates and 2 patients infected with fully-resistant *Candida* strains (40, 80). Baddley et al. reported a similar pattern of increasing all-cause mortality in 96 patients who had daily fluconazole dose/MIC ratios of <11.5, although overall mortality rates were similar between patients infected with *C. albicans* (35.7%) and *C. krusei* (40%) and were considerably lower in patients with *C. glabrata* (15.8%) (11).

More recently, Klevay and colleagues examined the relationship of initial treatment and clinical outcome for patients with *C. glabrata* versus *C. albicans* bloodstream infection in the Prospective Antifungal Therapy (PATH) multicenter observational registry data set (53, 54). Patients with *C. albicans* and *C. glabrata* bloodstream infection were matched 1:1 ($n = 161$ each) based on baseline clinical characteristics and compared with respect to time to initiation of appropriate antifungal therapy, in vitro antifungal resistance, and mortality. Patients with *C. glabrata* were more likely to have received inadequate initial antifungal therapy (34% versus 11%; $P < 0.05$), but 4-week mortality was no different between the two groups (30% for *C. glabrata* versus 29% for *C. albicans*) (54). Unlike previous studies, the authors were unable to demonstrate a trend in mortality in relation to the timing of antifungal therapy in their cohort of patients who were closely matched based on underlying severity of illness (53, 54). The only factors that were predictive of mortality at 4 weeks included presence of underlying hematologic malignancy, age of >60 years, presence of a central venous catheter at the time of diagnosis, and requirements for mechanical ventilation or renal replacement therapy (54).

Collectively, these studies suggest that while timing and appropriateness of antifungal therapy are probably important, their impact on the survival of patients with candidemia is often overshadowed by the underlying disease severity of the host (7, 83). In fact, data from Klevay et al. suggested that the analysis of survival differences in patients treated with adequate versus inadequate antifungal therapy from previous studies may be heavily biased by the inclusion of patients in study cohorts who are not aggressively treated because of a poor prognosis for underlying conditions, or patients who never receive antifungal therapy for candidemia (53, 54). Typically, untreated candidemic patients fall into one of several categories: (i) patients who have died shortly after the time yeast is identified in blood cultures, (ii) patients who are being transitioned to palliative care only, (iii) patients with inappropriate characterization of the positive blood culture as contamination, (iv) patients for whom there was a mistaken belief that candidemia was effectively treated with removal of the central venous catheter alone because the patient appeared to be clinically stable, and (v) rare patients for whom therapy was withheld until the blood culture could be positively identified (53). Delays in treatment may also reflect an underrecognition of candidemia in chronically ill outpatients (3, 19, 44, 46).

Resistance and Pathogen Virulence

An alternative hypothesis to explain the similarity in outcome of infections due to antifungal-susceptible and -resistant *Candida* species is that antifungal-resistant species are generally less virulent than antifungal-susceptible strains (33, 53). This concept is supported by comparative virulence studies performed by Arendrup and colleagues with nonimmunocompromised mice where lethal infection was only observed after intravenous challenge with fluconazole-susceptible *C. albicans* and *C. tropicalis* (8). Based on survival, organ fungal burden, and histopathological analysis, *Candida* isolates could be divided into three groups with decreasing virulence: (i) *C. albicans* and *C. tropicalis*; (ii) *C. glabrata, C. kefyr*, and *C. lusitaniae*; and (iii) *C. parapsilosis*, *C. krusei*, and *C. guilliermondii* (8). Notably, the authors found that two or three isolates within each species behaved identically in the model, making it reasonable to pool the results for each species. Moreover, the virulence ranking from the model correlated with the clinical incidence in species reported from a survey of 1,591 patients with systemic *Candida* infection in which *C. albicans* accounted for 54% of cases, followed by *C. tropicalis* (25%), *C. glabrata* (8%), *C. parapsilosis* (7%), and *C. krusei* (8%) (8, 83). Although it is common in retrospective studies to report higher crude mortality rates with *C. glabrata* and *C. krusei* bloodstream infections (48, 112, 116), these less virulent opportunistic species are more likely to emerge in the severely immunosuppressed, older, or critically ill patient, thus overshadowing pathogen-driven differences in the severity of infection. Interestingly, mixed candidemias (infections caused by two or more species) have remained relatively constant in most cases series, at ≤5% (7, 50, 110).

Relatively less is known about the impact of acquired antifungal resistance (through genetic mutations) on virulence in *Canidida* species. In bacteria and fungi, the emergence of antimicrobial resistance is often associated with fitness costs and thus results in a competitive disadvantage against otherwise drug-susceptible organisms within the host, although compensatory mechanisms can arise that alleviate the fitness cost of resistance (4, 26). Graybill and colleagues examined this question in *C. albicans* by testing the virulence of azole-resistant isolates in a mouse model of invasive candidiasis compared to their azole-susceptible parental isolate (42). The authors found no direct relationship between

fluconazole susceptibility and survival (virulence) in their model.

In contrast, Ferrari and colleagues recently found that gain-of-function mutations in the transcriptional regulator CgPDR1—the key modulator of multidrug efflux pump expression in *C. glabrata*—were associated not only with higher levels of in vitro and in vivo resistance to fluconazole but also increased virulence and "dominance" of the fungal population in mice even in the absence of drug selection (35). Although this paper gives perhaps the first example of in vivo-acquired mutations in a fungal gene with a positive impact on in vivo fitness, all of the clinical isolates used in the study were acquired from semi-invasive infections of the oropharynx rather than the bloodstream of patients. In contrast, clinical and laboratory studies examining the virulence of echinocandin-resistant *C. albicans* and *C. tropicalis* have suggested that mutations in the protein target for echinocandin antifungals that leads to resistance, Fks1p, may be associated with impaired hypha formation, altered biofilm responses, and decreased virulence in vivo (13, 60). We have anecdotally observed that bloodstream infections with echinocandin-resistant strains of *C. albicans* and *C. tropicalis* often present as less fulminant infections in leukemia patients with hematogenous candidiasis, compared to more severe infections with echinocandin-susceptible strains (39). Therefore, it is possible that the cost of acquired resistance on *Candida* virulence varies among different *Candida* isolates and drug classes. Alternatively, some resistant isolates may incite less vigorous inflammatory responses without distinct differences in virulence (20).

IS THE PROBLEM OF ANTIFUNGAL RESISTANCE IN *CANDIDA* SPECIES INCREASING?

Intrinsic Antifungal Resistance in *Candida* Species

Candida species account for roughly 75% of all invasive fungal infections in hospitalized patients, with five species causing approximately 95% of all infections: *C. albicans*, *C. tropicalis*, *C. glabrata*, *C. krusei*, *and C. parapsilosis* (92, 96). Of these five species, only *C. krusei* is considered to be intrinsically resistant to fluconazole, but it is often susceptible to other azoles such as voriconazole and posaconazole (98). Rates of *C. krusei* infection have remained relatively stable over the last two decades, representing 1 to 5% of all *Candida*-associated bloodstream infections, and they are generally restricted to patients with acute leukemias who develop mucositis and prolonged neutropenia following remission induction chemotherapy (98) (Fig. 1). In an analysis of geographic and temporal trends from the ARTEMIS surveillance program, Pfaller and colleagues reported that the overall frequency of *C. krusei* across North America, the European Union, Africa and the Middle East, and Asian-Pacific was unchanged from 2001 to 2005, ranging from 2.3 to 2.7% (97). The majority of isolates came from hematology-oncology patients (97). Overall, 83% of the *C. krusei* isolates were susceptible to voriconazole, with no evidence of increasing resistance over the 5-year period (97). All isolates were classified as susceptible to the echinocandins. Interestingly, decreasing susceptibilities to amphotericin B (MIC_{90}, 4 μg/ml) and flucytosine (MIC_{90}, 16 μg/ml) were observed over the study period.

Although not as uniformly resistant as *C. krusei*, *C. glabrata* is the most common non-fluconazole-susceptible *Candida* species encountered in patients that affects empirical therapy choices (17, 48, 82). In an analysis of 23,305 clinical *C. glabrata* isolates collected during the ARTEMIS surveillance program, rates of fluconazole resistance ranged from 6 to 30% depending on the region or country (95) (Fig. 1). Multilocus sequence typing of diverse isolate collections has suggested that the variations in resistance rates may reflect distinct genetic clades of *C. glabrata* that prevail in certain geographic regions (29), and possibly host and geographic differences in the fungal microbiome (41). In the United States, the proportion of *C. glabrata* fungemia varies from 11 to 37%, with the highest frequency of fluconazole-resistant isolates in the northeastern (17%), midwestern (12%), and southern (11%) portions of the country (95). Overall, the incidence of *C. glabrata* infections in Latin America, the European Union, Africa and the Middle East, and the Asian-Pacific countries was lower, ranging from 5 to 13% (97).

Temporally, the overall incidence of fluconazole resistance among *C. glabrata* isolates increased significantly in those collected from 2001 to 2007 compared to 1992 to 2001 (97). Yet while the frequency of *C. glabrata* isolation appeared to increase with patient age, rates of fluconazole resistance declined in older adults. For example, patients ≥80 years of age had the highest proportion of bloodstream infections due to *C. glabrata* (32%) but the lowest rate of fluconazole resistance (5%) (101). More recent analysis of *Candida* susceptibility from the SENTRY Antimicrobial Surveillance Program (2008 to 2009) suggests that the highest rates of resistance to echinocandins (16.7%), fluconazole (16.7%), posaconazole (5.0%), and voriconazole (11.1%) are found among *C. glabrata* isolates recovered from 20- to 39-year-old adults (95). Indeed, *C glabrata* isolates with MDR phenotypes appeared to be concentrated in the 20- to 39-year age group (95). Therefore, an increasingly aged population may be driving the higher overall frequency of *C. glabrata* infections in U.S. hospitals, but the prevalence of antifungal-resistant strains is increasingly concentrated in relatively younger patients (93, 95).

C. parapsilosis is the third or fourth most common *Candida* species encountered in most medical centers. *C. parapsilosis* is frequently associated with catheter-related bloodstream infections due to its well-recognized ability to form dense biofilms (61, 84, 115). *C. parapsilosis* bloodstream infections are associated with lower mortality rates compared to other *Candida* species, and antifungal resistance, particularly to fluconazole, is still uncommon (48, 83, 115). Wild-type *C. parapsilosis* isolates are less susceptible to echinocandins due to naturally occurring polymorphisms in the *FKS* target of the glucan synthase inhibitors (38, 88). Reports of increased incidence of breakthrough infections on echinocandin therapy in individual hospitals and in U.S.-based population surveillance programs (28, 46, 99), as well as the widespread use of echinocandins as front-line therapy for invasive candidiasis (37, 48, 112), have fueled concerns of increasing resistance in *C. parapsilosis* (6). However, contemporary surveillance programs have not provided any evidence of increasing resistance among this species (21, 87, 91, 99), nor have *C. parapsilosis* isolates with higher MICs been associated with poorer treatment responses to echinocandin therapy in prospective clinical trials (52).

Among the other 15 to 18 species of *Candida* known to cause invasive candidiasis, several rare species are of interest because they have been shown to cause clusters of nosocomial infection, have been increasing in frequency, or exhibit decreased in vitro susceptibility to one or more classes of

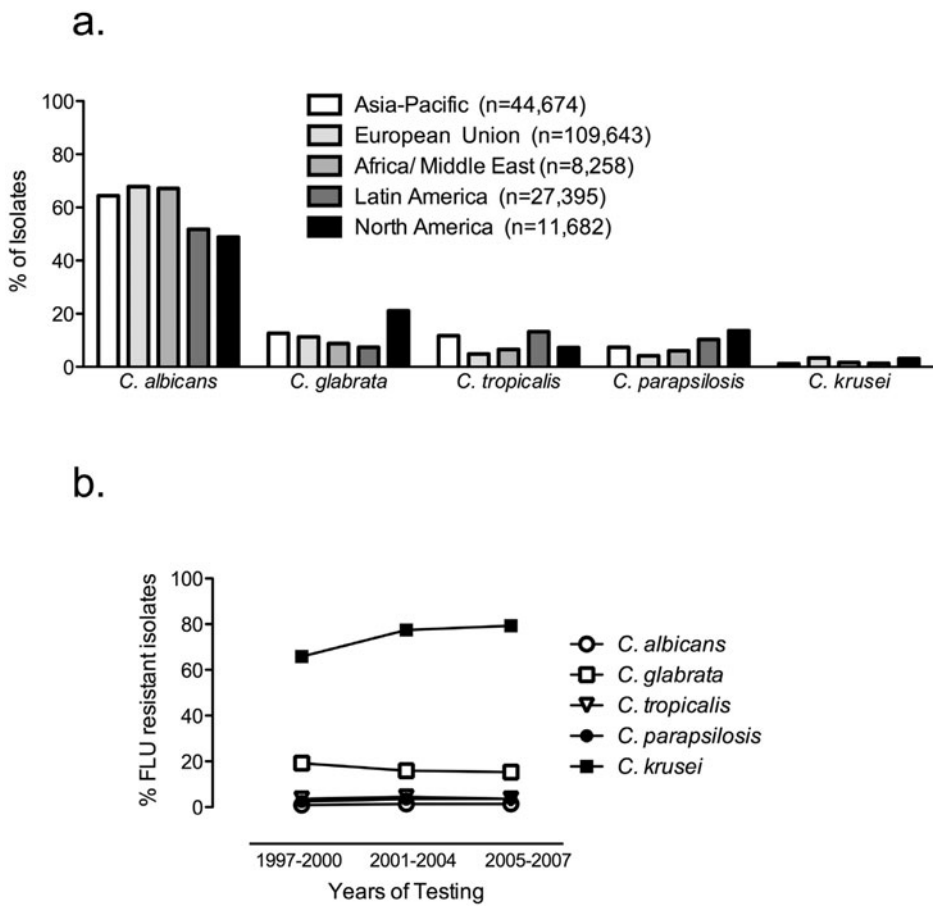

FIGURE 1 Geographic variation in (a) *Candida* species and (b) rates of fluconazole resistance reported from the ARTEMIS Global Surveillance Program, 2001 to 2007 (97). 10.1128/9781555817176.ch23f1

antifungal agents (27). *C. lusitaniae* has been a pathogen of interest due to its propensity to cause breakthrough fungemia in patients with malignancies or other serious comorbid conditions, as well as its capacity to rapidly develop resistance to amphotericin B during the course of therapy (9, 47, 67). Likewise, *C. guilliermondii* and *C. rugosa* are uncommon species of *Candida* that are occasionally responsible for clusters of fungemia in patients hospitalized for cancer treatment or in medical and surgical intensive care units (ICUs). *C. guilliermondii* and *C. rugosa* often display reduced decreased susceptibility to amphotericin B, fluconazole, and the echinocandins (89, 94, 103). These species continue to be associated with sporadic infections that are occasionally difficult to treat if they involve a foreign body that cannot be removed (e.g., a central venous catheter) or are localized to a sanctuary anatomical site where antifungal penetration is limited, such as the eyes or central nervous system (CNS) (31).

While these global trends are important to document, clinicians are primarily concerned about the local epidemiology of antifungal resistance in their community. A closer inspection of data from the ARTEMIS surveillance program (97) as well as recently published candidemia registry trials (48, 70, 71) reveals considerable heterogeneity in the isolation of antifungal-resistant *Candida* species across different regions, medical centers, and even different medical services within the same hospital. A number of reasons may account for this variability, including density of azole use, patient age, underlying disease and comorbidities, infection control practices at local institutions, geographic and environmental factors, and blood culture systems used for isolation (34, 92, 93). Clearly, fluconazole resistance is a problem in high-risk transplant and oncology populations, but it is not a universal problem such as methicillin-resistant *Staphylococcus aureus* or MDR gram-negative bacteria (108). Likewise, susceptibility trends published in the medical literature generally reflect aggregate patterns of resistance observed from isolates collected at large tertiary-care medical centers, but these may be less relevant for smaller community hospitals that have lower rates of candidemia and fluconazole resistance (12, 18, 79). Indeed, many small community hospitals in the United States do not have an on-site clinical microbiology laboratory and identify yeast in blood cultures to the species level only at the request of the physician (79, 102). Consequently, antifungal resistance is often viewed (perhaps incorrectly) as a problem restricted to large medical centers.

Irrespective of the local epidemiology, the clinical question associated with intrinsic antifungal resistance in patients with invasive candidiasis remains the same: what is the likelihood that a particular patient is infected with an antifungal-resistant species? For reasons discussed earlier, the major concern throughout the world and most medical centers is fluconazole-resistant *C. glabrata*. Current treatment

guidelines for the management of invasive candidiasis endorsed by the Infectious Diseases Society of America recommend administration of appropriately dosed fluconazole in nonneutropenic patients with less severe illness, and where *C. glabrata* or *C. krusei* is unlikely (82). Echinocandins are generally preferred first-line therapy for patients with infection due to *C. glabrata* or *C. krusei* or for the treatment of candidemia in neutropenic patients (82, 117). Amphotericin B (including lipid formulations) is generally reserved as second-line therapy, but many experts still recommend its initial use in patients with profound cell-mediated immunodeficiency who are at risk for infection with *Cryptococcus neoformans* or endemic fungi, which cannot be treated with echinocandins (55, 77). Although not specifically studied in clinical trials, the current treatment guidelines endorse step-down therapy to an oral fluconazole to complete at least 2 weeks of treatment if the patient is clinically responding and can take oral therapy (82).

While these consensus-driven treatment approaches are sensible for most patients, in practice it can be very difficult to predict which patients are infected with resistant species. The association of prior azole use and the increased isolation of *C. glabrata* is well known (1, 2, 36, 45, 85, 104, 113), but evidence for this link is primarily derived from cancer hospitals, where many patients receive prolonged courses of fluconazole prophylaxis (65, 95, 107). Multiple single-center retrospective studies with primarily nonneutropenic noncancer patients have suggested that besides older age, prior exposure to broad-spectrum antimicrobials (but not necessarily fluconazole) is the predisposing risk factor for *C. glabrata* infection (63, 78, 95). Shorr and colleagues examined whether clinical features in nonneutropenic hospitalized patients could be used to develop a predictive model of identifying patients at higher risk for infection with fluconazole-resistant *C. glabrata* or *C. krusei* (111). The investigators found that although non-*C. albicans Candida* species were common in their wards and ICUs (including fluconazole-resistant strains), no clinical factors could be identified that allow the clinician to effectively identify patients infected with non-*C. albicans* or fluconazole-resistant fungi (110). Consequently, many clinicians now use echinocandins as empirical therapy until isolates are identified to the species level and, in some cases, antifungal susceptibility is confirmed. This strategy may be prudent, as Oxman and colleagues found that among fluconazole-resistant isolates identified at two U.S. hospitals, *C. albicans*, *C. tropicalis*, and *C. parapsilosis* accounted for 48% of the fluconazole-resistant strains (78). Prior exposure to antifungal agents was noted to be a key risk factor for fluconazole resistance in these typically susceptible species (odds ratio, 2.2; $P = 0.04$) (78).

Acquired Antifungal Resistance in *Candida* Species

Candida species can develop resistance to antifungal agents through a variety of mechanisms that can result in treatment failure (Fig. 2) (57, 108). However, there are important differences between resistance development and its transmission in fungi versus bacteria. Fungi generally do not have mechanisms comparable to those of bacteria for transferring extrachromosomal resistance genes (e.g., transposons), and transformation of diploid fungi with DNA is generally more difficult than with bacteria (108). One exception is *C. glabrata*, which can easily acquire high-level resistance to triazoles due to a high rate of genetic mutation in vivo and readily express these resistance mechanisms due to the haploid nature of its genome (23, 36). Yet for most clinicians, the threat of acquired antifungal resistance may seem relatively benign compared to bacterial pathogens, or at least more predictable. Breakthrough infections on previously effective antifungal therapy occur most frequently with sustained exposure to antifungals, such as among patients in the pre-highly active antiretroviral therapy era with advanced AIDS and recurrent oropharyngeal candidiasis (66). In the hospital, well-documented cases of acquired resistance with *Candida* typically arise in the setting of suboptimal source control of the infection (i.e., retained prosthetic material, infected valve, or undrained fluid collection) or persistent neutropenia that necessitates continuous antifungal therapy that eventually fails to control the infection (17, 48, 58, 76). However, transmission of these resistant strains to untreated individuals does not seem to occur on any significant scale (36).

Clinical Resistance as a Cause of Treatment Failure

The majority of patients with invasive mycoses probably fail therapy because of underlying host factors, rather than acquired resistance to the drug (51). The concept of clinical resistance in patients with candidemia refers to the interplay of host- and pathogen-specific factors that affect clinical outcome but cannot be directly linked to the MIC of the pathogen (51). Common circumstances that contribute to clinical resistance are described in Table 2. While some of these factors are directly drug related (e.g., inadequate dosing, drug interactions, and toxicity) and can often be corrected, many host-related factors may require days to weeks before improvement is observed, if at all. One of the most underappreciated causes of treatment failures in *Candida* species is biofilm-mediated resistance. *C. albicans* biofilm cells are phenotypically distinct and often capable of surviving antifungal concentrations up to 1,000-fold higher than planktonic cells (10, 30). Interestingly, biofilm exopolysaccharides, including β-1,3 D-glucan, can directly bind amphotericin B and fluconazole, which dramatically limits their penetration and activity against cells embedded in the polysaccharide matrix (72–75). In contrast, lipid amphotericin B formulations and echinocandins are not extensively inactivated by the biofilm expopolysaccharides, and they appear to retain activity against biofilm-embedded cells (59). However, it is unknown whether in vitro differences in biofilm-mediated resistance among the triazoles, lipid amphotericin B formulations, and echinocandins translate to clinically important differences.

IS ANTIFUNGAL SUSCEPTIBILITY TESTING USEFUL FOR GUIDING TREATMENT DECISIONS?

Until the last decade, techniques for antifungal susceptibility testing were not well standardized, making it difficult to evaluate the relationship between MIC test results and clinical outcome. The Clinical and Laboratory Standards Institute (CLSI) developed and published reference methods for susceptibility testing of yeast that culminated with the M27-A3 microdilution (25) and the M44-A disk testing (69) methods, as well as interpretive susceptibility breakpoints for triazoles and echinocandins against *Candida species* (100, 105). Similar testing guidelines have been proposed by the European Committee on Antibiotic Susceptibility Testing (32, 90). Overall, the standardized methods

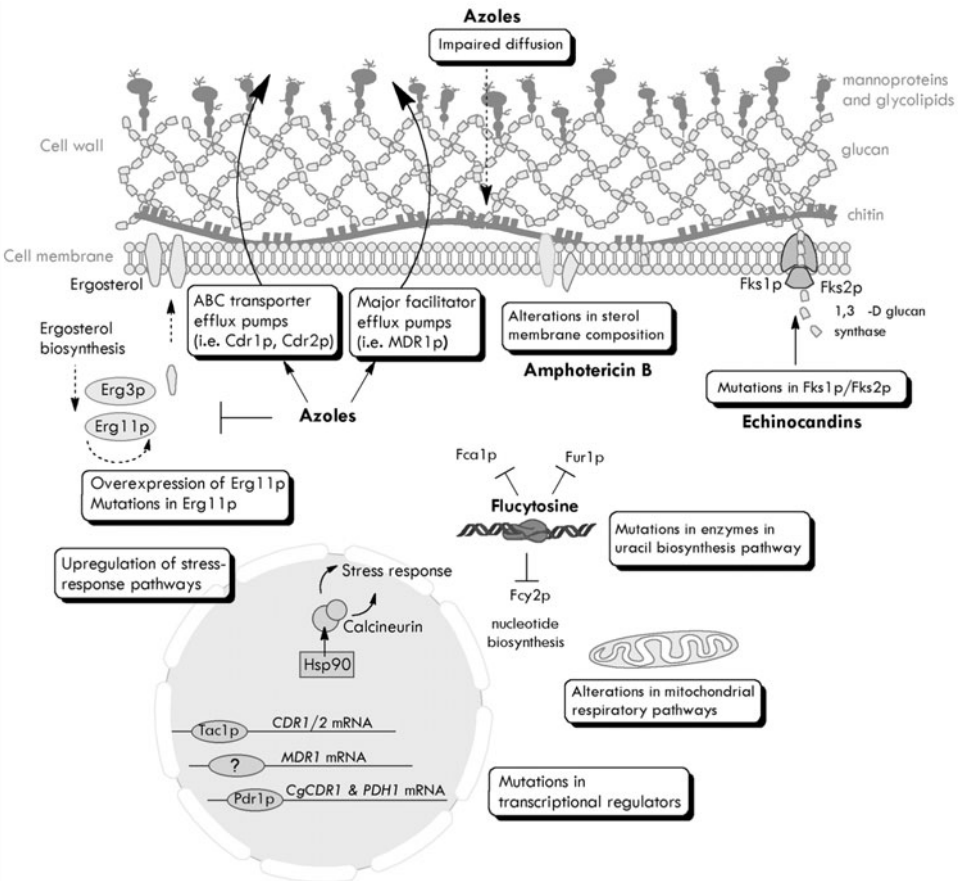

FIGURE 2 Antifungal resistance mechanisms in *Candida* species. 10.1128/9781555817176.ch23f2

and interpretive criteria provide similar rates of clinical correlation that are achieved with antibacterial susceptibility testing (105, 106), although the strongest correlation of antifungal MICs and clinical failure are observed with fluconazole in the treatment of oropharyngeal candidiasis and possibly bloodstream candidiasis (5, 106).

Current Infectious Diseases Society of America treatment guidelines recommend that laboratories provide routine antifungal susceptibility testing against fluconazole for *C. glabrata* isolates recovered from blood and sterile sites and for other *Candida* species that have failed antifungal therapy or in which azole resistance is strongly suspected (82). A survey of antifungal susceptibility testing practices in U.S. teaching hospitals found that roughly two-thirds offered susceptibility testing of *Candida* isolates from blood or sterile sites, but 76% of these centers sent the samples off-site for testing (79). The median time to obtain susceptibility results was significantly lower when testing was performed on-site (3 days) versus off-site (7 to 10 days) (34, 79); in addition, 3 to 4 days was initially required for species identification (34, 79). Consequently, many physicians do not receive antifungal susceptibility test information until near the end of a full treatment course for uncomplicated candidemia, giving support to the sentiment that antifungal MIC is often an abbreviation for "meaningless information for clinicians!"

The indifferent attitude of many clinicians to antifungal susceptibility testing is also no doubt supported by the reality that in many hospitals the susceptibility of particular *Candida* species to currently available antifungal agents is generally predictable (82). Yet individual isolates do not always follow these patterns (78), and antifungal susceptibility patterns (through systematic testing and reporting as an antifungal hospital-specific antibiogram) and individualized testing are becoming increasingly important in the management of invasive candidiasis. The emergence of MDR *C. glabrata* and echinocandin resistance, while such resistance is currently rare (95), could further increase the need for timely susceptibility testing in treatment algorithms of invasive candidiasis.

IS MOLECULAR TESTING THE ANSWER?

Problems associated with timely diagnosis and early detection of antifungal resistance in *Candida* species have not improved over the last two decades, as current testing approaches still rely primarily on blood cultures, which may be negative for up to 50% of patients (14, 24). These limitations have driven interest in the use of non-culture-based assays for detection of *Candida* antigens in serum (i.e., β-1, 3-D-glucan assay), as well as molecular techniques (i.e., peptide nucleic acid fluorescent in situ hybridization [PNA-FISH]) that can more rapidly distinguish *C. albicans* from non-*C. albicans Candida* species (109, 120). Although the PNA-FISH assay provides presumptive information on species more rapidly than the conventional germ tube

TABLE 2 Circumstances contributing to clinical resistance in patients with invasive candidiasis[a]

Risk factor	Implications
Erroneous diagnosis	Lack of specific diagnosis, especially with empirical or preemptive therapy; immune-reconstitution inflammatory syndromes, especially with recovery of neutropenia Patient may be infected with multiple pathogens (fungal and/or bacterial).
Persistently severe net state of immunosuppression	Antifungal therapy cannot control unopposed fungal proliferation in vivo in the setting of severe persistent immunodeficiencies, such as those that occur with graft failure or poorly controlled leukemia or persistent neutropenia and steroid-refractory graft-versus-host disease.
Poor underlying organ function	Renal and hepatic dysfunction and need for mechanical ventilation increase risk of drug toxicities and may limit aggressiveness of treatment measures.
Lack of source control	Failure to remove infected central venous catheters is associated with increased risk of death and treatment failure (i.e., severe thrombocytopenia preventing removal of implanted catheters). Fungemia often persists despite antifungal therapy in patients with undrained fluid collections or fistulas in the abdomen or with infected heart valves.
Delayed diagnosis	Increased risk of dissemination to visceral organs and sanctuary sites (such as the CNS or eyes) where antifungal penetration is impaired
Site of infection	Echinocandins, voriconazole, itraconazole, and posaconazole have limited activity against *Candida* spp. in the urine. Breakthrough fungal infections in anatomic sanctuary sites (such as the CNS or vitreous fluid) or infected hardware where antifungal penetration is limited *Candida* forms thick biofilms on central venous catheters and mucosal membranes that directly impair the activity of triazoles and amphotericin B. Lipid formulations of amphotericin B and echinocandins have improved activity against biofilm-embedded organisms. Risk of patient mortality is increased with retention of infected catheter.
Suboptimal treatment doses and duration of therapy	Inadequate dosing of fluconazole has been identified as a independent risk factor for patient mortality in invasive candidiasis, particularly the frequent use of small fixed doses (i.e., 200 or 400 mg/day) in increasingly obese populations. Administration of fluconazole without a loading dose, or at daily intervals in pediatric patients with rapid clearance, may result in inadequate drug exposures. Treatment courses of <2 wks for even uncomplicated candidemia have been associated with increased risk of relapse.
Drug toxicities and drug interactions	Nephrotoxicity (polyenes), hepatitis (triazoles), and pharmacokinetic drug-drug interactions (caspofungin or triazoles) may require treatment interruptions or result in ineffective blood levels.

[a]Adapted with modifications from reference 51.

test in serum or specialized identification media such as CHROMagar, the assay is considerably more expensive (81). Therefore, most laboratories still rely on a battery of biochemical tests offered in the API-20C strip to identify *Candida* to the species level (81).

A number of assays that involve detection and/or amplification of fungal nucleic acids using a range of amplicon detection formats (e.g., sequenced-based, real-time PCR and nucleic acid sequence-based amplification) have been developed to improve the detection and identification of pathogens from blood culture bottles or directly from patient blood samples and clinical specimens (24). The diagnostic potential of panfungal PCR assays performed in *positive* blood cultures may be limited, however, as molecular identification may only precede culture by a few hours (24). *Candida*-specific PCR assays for positive culture would have greater clinical utility if they could simultaneously identify pathogens and incipient antifungal resistance mechanisms (86). Newer molecular probe technology has opened up possibilities in assay design that could allow for detection not only of the fungal pathogens but also of antifungal resistance mechanisms that result from genetic changes in drug target site genes, chromosomal aneuploidy, or genetic elements controlling expression of drug efflux transporters (86). These assays would have the greatest clinical impact if they could be performed directly on blood or clinical samples rather than waiting for positive cultures. The optimal approach to using molecular methods, as well as a number of technical factors requiring standardization for the detection of resistance mechanisms in infections associated with low microbial burden, remains to be resolved. However, these methods could potentially be cost-effective in a high-volume clinical microbiology laboratory that services hospitals with large populations at risk for invasive fungal infections.

SUMMARY

There is little doubt that antifungal resistance in the clinic can contribute to treatment failure for invasive candidiasis. However, the currently low background rates of resistance among safe and effective first-line treatment options such as the echinocandins, relatively inefficient dissemination of acquired resistance among fungi versus bacteria, and overriding importance of underlying host factors in the outcome of *Candida* bloodstream infections create a perception among many clinicians that concerns surrounding antifungal resistance are a little overstated. In an era when microbiology resources in hospitals are limited, it is understandable that priority would be shifted towards the dire problems

of MDR bacterial pathogens at the expense of detecting and reporting antifungal resistance. Yet drug resistance is a problem that is always best to address by looking forward rather than backwards, and there are many signs on the horizon that antifungal resistance will become an even greater challenge in the next decade. Therefore, it is critical that the diagnostics and treatment tools to manage antifungal resistance in *Candida* species be developed now, along with clinical stewardship programs to effectively integrate their use in clinic, so we are better equipped to deal with the challenges on the horizon that will be encountered in an increasingly complex and aged population of patients in the health care system.

REFERENCES

1. **Abi-Said, D., E. Anaissie, O. Uzun, I. Raad, H. Pinzcowski, and S. Vartivarian.** 1997. The epidemiology of hematogenous candidiasis caused by different *Candida* species. *Clin. Infect. Dis.* **24:**1122–1128.
2. **Alexander, B. D., W. A. Schell, J. L. Miller, G. D. Long, and J. R. Perfect.** 2005. *Candida glabrata* fungemia in transplant patients receiving voriconazole after fluconazole. *Transplantation* **80:**868–871.
3. **Almirante, B., D. Rodriguez, B. J. Park, M. Cuenca-Estrella, A. M. Planes, M. Almela, J. Mensa, F. Sanchez, J. Ayats, M. Gimenez, P. Saballs, S. K. Fridkin, J. Morgan, J. L. Rodriguez-Tudela, D. W. Warnock, and A. Pahissa.** 2005. Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. *J. Clin. Microbiol.* **43:**1829–1835.
4. **Anderson, J. B.** 2005. Evolution of antifungal-drug resistance: mechanisms and pathogen fitness. *Nat. Rev. Microbiol.* **3:**547–556.
5. **Andes, D.** 2006. Pharmacokinetics and pharmacodynamics of antifungals. *Infect. Dis. Clin. N. Am.* **20:**679–697.
6. **Andes, D., D. J. Diekema, M. A. Pfaller, J. Bohrmuller, K. Marchillo, and A. Lepak.** 2010. In vivo comparison of the pharmacodynamic targets for echinocandin drugs against *Candida* species. *Antimicrob. Agents Chemother.* **54:**2497–2506.
7. **Antoniadou, A., H. A. Torres, R. E. Lewis, J. Thornby, G. P. Bodey, J. J. Tarrand, X. Y. Han, K. V. I. Rolston, A. Safdar, I. I. Raad, and D. P. Kontoyiannis.** 2003. Candidemia in a tertiary care cancer center. In vitro susceptibility and its association with outcome of initial antifungal therapy. *Medicine* **82:**309–321.
8. **Arendrup, M., T. Horn, and N. Frimodt-Moller.** 2002. In vivo pathogenicity of eight medically relevant *Candida* species in an animal model. *Infection* **30:**286–291.
9. **Atkinson, B. J., R. E. Lewis, and D. P. Kontoyiannis.** 2008. *Candida lusitaniae* fungemia in cancer patients: risk factors for amphotericin B failure and outcome. *Med. Mycol.* **46:**541–546.
10. **Bachmann, S. P., K. VandeWalle, G. Ramage, T. F. Patterson, B. L. Wickes, J. R. Graybill, and J. L. Lopez-Ribot.** 2002. In vitro activity of caspofungin against *Candida albicans* biofilms. *Antimicrob. Agents Chemother.* **46:**3591–3596.
11. **Baddley, J. W., M. Patel, S. M. Bhavnani, S. A. Moser, and D. R. Andes.** 2008. Association of fluconazole pharmacodynamics with mortality in patients with candidemia. *Antimicrob. Agents Chemother.* **52:**3022–3028.
12. **Baran, J., Jr., B. Muckatira, and R. Khatib.** 2001. Candidemia before and during the fluconazole era: prevalence, type of species and approach to treatment in a tertiary care community hospital. *Scand. J. Infect. Dis.* **33:**137–139.
13. **Ben-Ami, R., G. Garcia-Effron, R. E. Lewis, K. Leventakos, D. S. Perlin, and D. P. Kontoyiannis.** 2009. Mutations in *Candida albicans* FKS1 conferring echinocandin resistance are associated with attenuated virulence. *Abstr. 49th Intersci. Conf. Antimicrob. Agents Chemother.* American Society for Microbiology, Washington, DC.
14. **Berenguer, J., M. Buck, F. Witebsky, F. Stock, P. A. Pizzo, and T. J. Walsh.** 1993. Lysis-centrifugation blood cultures in the detection of tissue-proven invasive candidiasis. Disseminated versus single-organ infection. *Diagn. Microbiol. Infect. Dis.* **17:**103–109.
15. **Blot, S. I., and K. H. Vandewoude.** 2003. Estimating attributable mortality of candidemia: clinical judgement vs. matched cohort studies. *Eur. J. Clin. Microbiol. Infect. D* **22:**132–133.
16. **Blot, S. I., K. H. Vandewoude, E. A. Hoste, and F. A. Colardyn.** 2002. Effects of nosocomial candidemia on outcomes of critically ill patients. *Am. J. Med.* **113:**480–485.
17. **Bodey, G. P., M. Mardani, H. A. Hanna, M. Boktour, J. Abbas, E. Girgawy, R. Y. Hachem, D. P. Kontoyiannis, and I. I. Raad.** 2002. The epidemiology of *Candida glabrata* and *Candida albicans* fungemia in immunocompromised patients with cancer. *Am. J. Med.* **112:**380–385.
18. **Bustamante, C. I.** 2005. Treatment of *Candida* infection: a view from the trenches! *Curr. Opin. Infect. Dis.* **18:**490–495.
19. **Cano, M. V., J. F. Perz, A. S. Craig, M. Liu, G. M. Lyon, M. E. Brandt, T. J. Lott, B. A. Lasker, F. F. Barrett, M. M. McNeil, W. Schaffner, and R. A. Hajjeh.** 2005. Candidemia in pediatric outpatients receiving home total parenteral nutrition. *Med. Mycol.* **43:**219–225.
20. **Casadevall, A., and L. A. Pirofski.** 1999. Host-pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect. Immun.* **67:**3703–3713.
21. **Castanheira, M., L. N. Woosley, D. J. Diekema, S. A. Messer, R. N. Jones, and M. A. Pfaller.** 2010. Low prevalence of *fks1* hot spot 1 mutations in a worldwide collection of *Candida* strains. *Antimicrob. Agents Chemother.* **54:**2655–2659.
22. **Chamilos, G., M. Luna, R. E. Lewis, G. P. Bodey, R. Chemaly, J. J. Tarrand, A. Safdar, I. I. Raad, and D. P. Kontoyiannis.** 2006. Invasive fungal infections in patients with hematologic malignancies in a tertiary care cancer center: an autopsy study over a 15-year period (1989–2003). *Haematologica* **91:**986–989.
23. **Chapeland-Leclerc, F., C. Hennequin, N. Papon, T. Noel, A. Girard, G. Socie, P. Ribaud, and C. Lacroix.** 2010. Acquisition of flucytosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. *Antimicrob. Agents Chemother.* **54:**1360–1362.
24. **Chen, S. C., and D. P. Kontoyiannis.** 2010. New molecular and surrogate biomarker-based tests in the diagnosis of bacterial and fungal infection in febrile neutropenic patients. *Curr. Opin. Infect. Dis.* **23:**567–577.
25. **Clinical and Laboratory Standards Institute.** 2008. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts; Approved Standard—Third Edition.* Clinical and Laboratory Standards Institute, Wayne, PA.
26. **Cowen, L. E., L. M. Kohn, and J. B. Anderson.** 2001. Divergence in fitness and evolution of drug resistance in experimental populations of *Candida albicans*. *J. Bacteriol.* **183:**2971–2978.
27. **Diekema, D. J., S. A. Messer, L. B. Boyken, R. J. Hollis, J. Kroeger, S. Tendolkar, and M. A. Pfaller.** 2009. In vitro

activity of seven systemically active antifungal agents against a large global collection of rare *Candida* species as determined by CLSI broth microdilution methods. *J. Clin. Microbiol.* **47:**3170–3177.
28. **Diekema, D. J., S. A. Messer, R. J. Hollis, R. P. Wenzel, and M. A. Pfaller.** 1997. An outbreak of *Candida parapsilosis* prosthetic valve endocarditis. *Diagn. Microbiol. Infect. Dis.* **29:**147–153.
29. **Dodgson, A. R., C. Pujol, D. W. Denning, D. R. Soll, and A. J. Fox.** 2003. Multilocus sequence typing of *Candida glabrata* reveals geographically enriched clades. *J. Clin. Microbiol.* **41:**5709–5717.
30. **Donlan, R. M., and J. W. Costerton.** 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.* **15:**167–193.
31. **Dube, M. P., P. N. R. Heseltine, M. G. Rinaldi, S. Evans, and B. Zawacki.** 1994. Fungemia and colonization with nystatin-resistant *Candida rugosa* in a burn unit. *Clin. Infect. Dis.* **18:**77–82.
32. **Espinel-Ingroff, A., F. Barchiesi, M. Cuenca-Estrella, M. A. Pfaller, M. Rinaldi, J. L. Rodriguez-Tudela, and P. E. Verweij.** 2005. International and multicenter comparison of EUCAST and CLSI M27-A2 broth microdilution methods for testing susceptibilities of *Candida* spp. to fluconazole, itraconazole, posaconazole, and voriconazole. *J. Clin. Microbiol.* **43:**3884–3889.
33. **Falagas, M. E., K. E. Apostolou, and V. D. Pappas.** 2006. Attributable mortality of candidemia: a systematic review of matched cohort and case-control studies. *Eur. J. Clin. Microbiol. Infect. Dis.* **25:**419–425.
34. **Fernandez, J., B. L. Erstad, W. Petty, and D. E. Nix.** 2009. Time to positive culture and identification for *Candida* blood stream infections. *Diagn. Microbiol. Infect. Dis.* **64:**402–407.
35. **Ferrari, S., F. Ischer, D. Calabrese, B. Posteraro, M. Sanguinetti, G. Fadda, B. Rohde, C. Bauser, O. Bader, and D. Sanglard.** 2009. Gain of function mutations in CgPDR1 of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. *PLoS Pathog.* **5:** e1000268.
36. **Fidel, P. L., Jr., J. A. Vazquez, and J. D. Sobel.** 1999. *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin. Microbiol. Rev.* **12:**80–96, CP2.
37. **Forrest, G. N., E. Weekes, and J. K. Johnson.** 2008. Increasing incidence of *Candida parapsilosis* candidemia with caspofungin usage. *J. Infect.* **56:**126–129.
38. **Garcia-Effron, G., S. K. Katiyar, S. Park, T. D. Edlind, and D. S. Perlin.** 2008. A naturally occurring proline-to-alanine amino acid change in Fks1p in *Candida parapsilosis*, *Candida orthopsilosis*, and Candida *metapsilosis* accounts for reduced echinocandin susceptibility. *Antimicrob. Agents Chemother.* **52:**2305–2312.
39. **Garcia-Effron, G., D. P. Kontoyiannis, R. E. Lewis, and D. S. Perlin.** 2008. Caspofungin-resistant *Candida tropicalis* strains causing breakthrough fungemia in patients at high risk for hematologic malignancies. *Antimicrob. Agents Chemother.* **52:**4181–4183.
40. **Garey, K. W., M. Rege, M. P. Pai, D. E. Mingo, K. J. Suda, R. S. Turpin, and D. T. Bearden.** 2006. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. *Clin. Infect. Dis.* **43:**25–31.
41. **Ghannoum, M. A., R. J. Jurevic, P. K. Mukherjee, F. Cui, M. Sikaroodi, A. Naqvi, and P. M. Gillevet.** 2010. Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathog.* **6:** e1000713.
42. **Graybill, J. R., E. Montalbo, W. R. Kirkpatrick, M. F. Luther, S. G. Revankar, and T. F. Patterson.** 1998. Fluconazole versus *Candida albicans*: a complex relationship. *Antimicrob. Agents Chemother.* **42:**2938–2942.
43. **Gudlaugsson, O., S. Gillespie, K. Lee, J. V. Berg, J. F. Hu, S. Messer, L. Herwaldt, M. Pfaller, and D. Diekema.** 2003. Attributable mortality of nosocomial candidemia, revisited. *Clin. Infect. Dis.* **37:**1172–1177.
44. **Gulia, J., S. Aryal, H. Saadlla, and A. F. Shorr.** 2010. Healthcare-associated candidemia—a distinct entity? *J. Hosp. Med.* **5:**298–301.
45. **Hachem, R., H. Hanna, D. Kontoyiannis, Y. Jiang, and I. Raad.** 2008. The changing epidemiology of invasive candidiasis: *Candida glabrata* and *Candida krusei* as the leading causes of candidemia in hematologic malignancy. *Cancer* **112:**2493–2499.
46. **Hajjeh, R. A., A. N. Sofair, L. H. Harrison, G. M. Lyon, B. A. Arthington-Skaggs, S. A. Mirza, M. Phelan, J. Morgan, W. Lee-Yang, M. A. Ciblak, L. E. Benjamin, L. T. Sanza, S. Huie, S. F. Yeo, M. E. Brandt, and D. W. Warnock.** 2004. Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J. Clin. Microbiol.* **42:**1519–1527.
47. **Hawkins, J. L., and L. M. Baddour.** 2003. *Candida lusitaniae* infections in the era of fluconazole availability. *Clin. Infect. Dis.* **36:**E14–E18.
48. **Horn, D. L., D. Neofytos, E. J. Anaissie, J. A. Fishman, W. J. Steinbach, A. J. Olyaei, K. A. Marr, M. A. Pfaller, C. H. Chang, and K. M. Webster.** 2009. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. *Clin. Infect. Dis.* **48:**1695–1703.
49. **Ibrahim, E. H., G. Sherman, S. Ward, V. J. Fraser, and M. H. Kollef.** 2000. The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. *Chest* **118:**146–155.
50. **Jensen, J., P. Munoz, J. Guinea, M. Rodriguez-Creixems, T. Pelaez, and E. Bouza.** 2007. Mixed fungemia: incidence, risk factors, and mortality in a general hospital. *Clin. Infect. Dis.* **44:**e109–e114.
51. **Kanafani, Z. A., and J. R. Perfect.** 2008. Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. *Clin. Infect. Dis.* **46:**120–128.
52. **Kartsonis, N., J. Killar, L. Mixson, C. M. Hoe, C. Sable, K. Bartizal, and M. Motyl.** 2005. Caspofungin susceptibility testing of isolates from patients with esophageal candidiasis or invasive candidiasis: relationship of MIC to treatment outcome. *Antimicrob. Agents Chemother.* **49:** 3616–3623.
53. **Klevay, M. J., E. J. Ernst, J. L. Hollanbaugh, J. G. Miller, M. A. Pfaller, and D. J. Diekema.** 2008. Therapy and outcome of *Candida glabrata* versus *Candida albicans* bloodstream infection. *Diagn. Microbiol. Infect. Dis.* **60:** 273–277.
54. **Klevay, M. J., D. L. Horn, D. Neofytos, M. A. Pfaller, and D. J. Diekema.** 2009. Initial treatment and outcome of *Candida glabrata* versus *Candida albicans* bloodstream infection. *Diagn. Microbiol. Infect. Dis.* **64:**152–157.
55. **Kontoyiannis, D. P.** 2009. Echinocandin-based initial therapy in fungemic patients with cancer: a focus on recent guidelines of the Infectious Diseases Society of America. *Clin. Infect. Dis.* **49:**638–639. (Author's reply, **49:** 639–640.)
56. **Kontoyiannis, D. P., G. P. Bodey, and C. S. Mantzoros.** 2001. Fluconazole vs. amphotericin B for the management of candidaemia in adults: a meta-analysis. *Mycoses* **44:** 125–135.

57. **Kontoyiannis, D. P., and R. E. Lewis.** 2002. Antifungal drug resistance of pathogenic fungi. *Lancet* **359:** 1135–1144.
58. **Kovacicova, G., M. Lovaszova, J. Hanzen, A. Roidova, F. Mateicka, M. Lesay, and V. Krcmery.** 2001. Persistent fungemia—risk factors and outcome in 40 episodes. *J. Chemother.* **13:**429–433.
59. **Kuhn, D. M., T. George, J. Chandra, P. K. Mukherjee, and M. A. Ghannoum.** 2002. Antifungal susceptibility of *Candida* biofilms: unique efficacy of amphotericin B lipid formulations and echinocandins. *Antimicrob. Agents Chemother.* **46:**1773–1780.
60. **Kurtz, M. B., G. Abruzzo, A. Flattery, K. Bartizal, J. A. Marrinan, W. Li, J. Milligan, K. Nollstadt, and C. M. Douglas.** 1996. Characterization of echinocandin-resistant mutants of *Candida albicans*: genetic, biochemical, and virulence studies. *Infect. Immun.* **64:**3244–3251.
61. **Lattif, A. A., P. K. Mukherjee, J. Chandra, K. Swindell, S. R. Lockhart, D. J. Diekema, M. A. Pfaller, and M. A. Ghannoum.** 2010. Characterization of biofilms formed by *Candida parapsilosis*, *C. metapsilosis*, *and C. orthopsilosis*. *Int. J. Med. Microbiol.* **300:**265–270.
62. **Leroy, O., J. P. Gangneux, P. Montravers, J. P. Mira, F. Gouin, J. P. Sollet, J. Carlet, J. Reynes, M. Rosenheim, B. Regnier, and O. Lortholary.** 2009. Epidemiology, management, and risk factors for death of invasive *Candida* infections in critical care: a multicenter, prospective, observational study in France (2005-2006). *Crit. Care Med.* **37:**1612–1618.
63. **Lin, M. Y., Y. Carmeli, J. Zumsteg, E. L. Flores, J. Tolentino, P. Sreeramoju, and S. G. Weber.** 2005. Prior antimicrobial therapy and risk for hospital-acquired *Candida glabrata* and *Candida krusei* fungemia: a case-case-control study. *Antimicrob. Agents Chemother.* **49:**4555–4560.
64. **MacArthur, R. D., M. Miller, T. Albertson, E. Panacek, D. Johnson, L. Teoh, and W. Barchuk.** 2004. Adequacy of early empiric antibiotic treatment and survival in severe sepsis: experience from the MONARCS trial. *Clin. Infect. Dis.* **38:**284–288.
65. **Marr, K. A.** 2000. The changing spectrum of candidemia in oncology patients: therapeutic implications. *Curr. Opin. Infect. Dis.* **13:**615–620.
66. **Martins, M. D., M. Lozano-Chiu, and J. H. Rex.** 1998. Declining rates of oropharyngeal candidiasis and carriage of *Candida albicans* associated with trends toward reduced rates of carriage of fluconazole-resistant *C. albicans* in human immunodeficiency virus-infected patients. *Clin. Infect. Dis.* **27:**1291–1294.
67. **McClenny, N. B., H. H. Fei, E. J. Baron, A. C. Gales, A. Houston, R. J. Hollis, and M. A. Pfaller.** 2002. Change in colony morphology of *Candida lusitaniae* in association with development of amphotericin B resistance. *Antimicrob. Agents Chemother.* **46:**1325–1328.
68. **Morrell, M., V. J. Fraser, and M. H. Kollef.** 2005. Delaying the empiric treatment of candida bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. *Antimicrob. Agents Chemother.* **49:**3640–3645.
69. **National Committee for Clinical Laboratory Standards.** 2004. *Methods for Antifungal Disk Diffusion Susceptibility Testing of Yeasts; Approved Guideline M44-A*. National Committee for Clinical Laboratory Standards, Wayne, PA.
70. **Neofytos, D., J. A. Fishman, D. Horn, E. Anaissie, C. H. Chang, A. Olyaei, M. Pfaller, W. J. Steinbach, K. M. Webster, and K. A. Marr.** 2010. Epidemiology and outcome of invasive fungal infections in solid organ transplant recipients. *Transpl. Infect. Dis.* **12:**220–229.
71. **Neofytos, D., D. Horn, E. Anaissie, W. Steinbach, A. Olyaei, J. Fishman, M. Pfaller, C. Chang, K. Webster, and K. Marr.** 2009. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. *Clin. Infect. Dis.* **48:**265–273.
72. **Nett, J., and D. Andes.** 2006. *Candida albicans* biofilm development, modeling a host-pathogen interaction. *Curr. Opin. Microbiol.* **9:**340–345.
73. **Nett, J., L. Lincoln, K. Marchillo, and D. Andes.** 2007. Beta-1,3 glucan as a test for central venous catheter biofilm infection. *J. Infect. Dis.* **195:**1705–1712.
74. **Nett, J., L. Lincoln, K. Marchillo, R. Massey, K. Holoyda, B. Hoff, M. VanHandel, and D. Andes.** 2007. Putative role of beta-1,3 glucans in *Candida albicans* biofilm resistance. *Antimicrob. Agents Chemother.* **51:**510–520.
75. **Nett, J. E., K. M. Guite, A. Ringeisen, K. A. Holoyda, and D. R. Andes.** 2008. Reduced biocide susceptibility in *Candida albicans* biofilms. *Antimicrob. Agents Chemother.* **52:**3411–3413.
76. **Ostrosky-Zeichner, L., D. Kontoyiannis, J. Raffalli, K. M. Mullane, J. Vazquez, E. J. Anaissie, J. Lipton, P. Jacobs, J. H. van Rensburg, J. H. Rex, W. Lau, D. Facklam, and D. N. Buell.** 2005. International, open-label, noncomparative, clinical trial of micafungin alone and in combination for treatment of newly diagnosed and refractory candidemia. *Eur. J. Clin. Microbiol. Infect. Dis.* **24:** 654–661.
77. **Ostrosky-Zeichner, L., and P. G. Pappas.** 2006. Invasive candidiasis in the intensive care unit. *Crit. Care Med.* **34:**857–863.
78. **Oxman, D. A., J. K. Chow, G. Frendl, S. Hadley, S. Hershkovitz, P. Ireland, L. A. McDermott, K. Tsai, F. M. Marty, D. P. Kontoyiannis, and Y. Golan.** 2010. Candidaemia associated with decreased in vitro fluconazole susceptibility: is *Candida* speciation predictive of the susceptibility pattern? *J. Antimicrob. Chemother.* **65:**1450–1465.
79. **Pai, M. P., and S. L. Pendland.** 2003. Antifungal susceptibility testing in teaching hospitals. *Ann. Pharmacother.* **37:**192–196.
80. **Pai, M. P., R. S. Turpin, and K. W. Garey.** 2007. Association of fluconazole area under the concentration-time curve/MIC and dose/MIC ratios with mortality in nonneutropenic patients with candidemia. *Antimicrob. Agents Chemother.* **51:**35–39.
81. **Pappas, P. G.** 2006. Invasive candidiasis. *Infect. Dis. Clin. N. Am.* **20:**485–506.
82. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, and J. D. Sobel.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.
83. **Pappas, P. G., J. H. Rex, J. Lee, R. J. Hamill, R. A. Larsen, W. Powderly, C. A. Kauffman, N. Hyslop, J. E. Mangino, S. Chapman, H. W. Horowitz, J. E. Edwards, and W. E. Dismukes.** 2003. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin. Infect. Dis.* **37:**634–643.
84. **Pasqualotto, A. C., W. L. Nedel, T. S. Machado, and L. C. Severo.** 2006. Risk factors and outcome for nosocomial breakthrough candidaemia. *J. Infect.* **52:**216–222.
85. **Pasqualotto, A. C., R. A. Zimerman, S. H. Alves, V. R. Aquino, D. Branco, D. Wiltgen, A. do Amaral, R. Cechinel, S. M. Colares, I. G. da Rocha, L. C. Severo, and T.**

C. Sukiennik. 2008. Take control over your fluconazole prescriptions: the growing importance of *Candida glabrata* as an agent of candidemia in Brazil. *Infect. Control Hosp. Epidemiol.* **29:**898–899.
86. **Perlin, D. S.** 2009. Antifungal drug resistance: do molecular methods provide a way forward? *Curr. Opin. Infect. Dis.* **22:**568–573.
87. **Pfaller, M. A., L. Boyken, R. J. Hollis, J. Kroeger, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2008. In vitro susceptibility of invasive isolates of *Candida* spp. to anidulafungin, caspofungin, and micafungin: six years of global surveillance. *J. Clin. Microbiol.* **46:**150–156.
88. **Pfaller, M. A., L. Boyken, R. J. Hollis, J. Kroeger, S. A. Messer, S. Tendolkar, R. N. Jones, J. Turnidge, and D. J. Diekema.** 2010. Wild-type MIC distributions and epidemiological cutoff values for the echinocandins and *Candida* spp. *J. Clin. Microbiol.* **48:**52–56.
89. **Pfaller, M. A., L. Boyken, R. J. Hollis, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2006. In vitro susceptibilities of *Candida* spp. to caspofungin: four years of global surveillance. *J. Clin. Microbiol.* **44:**760–763.
90. **Pfaller, M. A., M. Castanheira, D. J. Diekema, S. A. Messer, G. J. Moet, and R. N. Jones.** 2010. Comparison of European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Etest methods with the CLSI broth microdilution method for echinocandin susceptibility testing of *Candida* species. *J. Clin. Microbiol.* **48:**1592–1599.
91. **Pfaller, M. A., M. Castanheira, S. A. Messer, G. J. Moet, and R. N. Jones.** 2010. Variation in *Candida* spp. distribution and antifungal resistance rates among bloodstream infection isolates by patient age: report from the SENTRY Antimicrobial Surveillance Program (2008-2009). *Diagn. Microbiol. Infect. Dis.* **68:**278–283.
92. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
93. **Pfaller, M. A., and D. J. Diekema.** 2010. Epidemiology of invasive mycoses in North America. *Crit. Rev. Microbiol.* **36:**1–53.
94. **Pfaller, M. A., D. J. Diekema, A. L. Colombo, C. Kibbler, K. P. Ng, D. L. Gibbs, and V. A. Newell.** 2006. *Candida rugosa*, an emerging fungal pathogen with resistance to azoles: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program. *J. Clin. Microbiol.* **44:**3578–3582.
95. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, R. Barton, H. Bijie, J. Bille, S. C. Chang, M. da Luz Martins, A. Duse, D. Dzierzanowska, D. Ellis, J. Finquelievich, I. Gould, D. Gur, A. Hoosen, K. Lee, N. Mallatova, M. Mallie, N. G. Peng, G. Petrikos, A. Santiago, J. Trupl, A. M. VanDen Abeele, J. Wadula, and M. Zaidi.** 2010. Geographic variation in the frequency of isolation and fluconazole and voriconazole susceptibilities of *Candida glabrata*: an assessment from the ARTEMIS DISK Global Antifungal Surveillance Program. *Diagn. Microbiol. Infect. Dis.* **67:**162–171.
96. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, H. Bijie, D. Dzierzanowska, N. N. Klimko, V. Letscher-Bru, M. Lisalova, K. Muehlethaler, C. Rennison, and M. Zaidi.** 2009. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: 10.5-year analysis of susceptibilities of noncandidal yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J. Clin. Microbiol.* **47:**117–123.
97. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, D. Ellis, V. Tullio, A. Rodloff, W. Fu, and T. A. Ling.** 2010. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *J. Clin. Microbiol.* **48:**1366–1377.
98. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, E. Nagy, S. Dobiasova, M. Rinaldi, R. Barton, and A. Veselov.** 2008. *Candida krusei*, a multidrug-resistant opportunistic fungal pathogen: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *J. Clin. Microbiol.***46:**515–521.
99. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, K. P. Ng, A. Colombo, J. Finquelievich, R. Barnes, and J. Wadula.** 2008. Geographic and temporal trends in isolation and antifungal susceptibility of *Candida parapsilosis*: a global assessment from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *J. Clin. Microbiol.* **46:**842–849.
100. **Pfaller, M. A., D. J. Diekema, L. Ostrosky-Zeichner, J. H. Rex, B. D. Alexander, D. Andes, S. D. Brown, V. Chaturvedi, M. A. Ghannoum, C. C. Knapp, D. J. Sheehan, and T. J. Walsh.** 2008. Correlation of MIC with outcome for *Candida* species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints. *J. Clin. Microbiol.* **46:**2620–2629.
101. **Pfaller, M. A., S. A. Messer, R. J. Hollis, L. Boyken, S. Tendolkar, J. Kroeger, and D. J. Diekema.** 2009. Variation in susceptibility of bloodstream isolates of *Candida glabrata* to fluconazole according to patient age and geographic location in the United States in 2001 to 2007. *J. Clin. Microbiol.* **47:**3185–3190.
102. **Pfaller, M. A., J. H. Rex, and M. G. Rinaldi.** 1996. Antifungal susceptibility testing: technical advances and potential clinical applications. *Clin. Infect. Dis.* **24:**776–784.
103. **Pfaller, M. A., S. Wey, T. Gerarden, A. Houston, and R. P. Wenzel.** 1989. Susceptibility of nosocomial isolates of *Candida* species to LY121019 and other antifungal agents. *Diagn. Microbiol. Infect. Dis.* **12:**1–4.
104. **Playford, E. G., D. Marriott, Q. Nguyen, S. Chen, D. Ellis, M. Slavin, and T. C. Sorrell.** 2008. Candidemia in nonneutropenic critically ill patients: risk factors for non-*albicans Candida* spp. *Crit. Care Med.* **36:**2034–2039.
105. **Rex, J. H., M. A. Pfaller, J. N. Galgiani, M. S. Bartlett, A. Espinel-Ingroff, M. A. Ghannoum, M. Lancaster, F. C. Odds, M. G. Rinaldi, T. J. Walsh, and A. L. Barry.** 1997. Development of interpretive breakpoints for antifungal susceptibility testing: conceptual framework and analysis of in vitro-in vivo correlation data for fluconazole, itraconazole, and candida infections. Subcommittee on Antifungal Susceptibility Testing of the National Committee for Clinical Laboratory Standards. *Clin. Infect. Dis.* **24:**235–247.
106. **Rex, J. H., M. A. Pfaller, T. J. Walsh, V. Chaturvedi, A. Espinel-Ingroff, M. A. Ghannoum, L. L. Gosey, F. C. Odds, M. G. Rinaldi, D. J. Sheehan, and D. W. Warnock.** 2001. Antifungal susceptibility testing: practical aspects and current challenges. *Clin. Microbiol. Rev.* **14:**643–658.
107. **Richet, H., P. Roux, C. Des Champs, Y. Esnault, and A. Andremont.** 2002. Candidemia in French hospitals: incidence rates and characteristics. *Clin. Microbiol. Infect.* **8:**405–412.
108. **Sanglard, D., and F. C. Odds.** 2002. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect. Dis.* **2:**73–85.
109. **Shepard, J. R., R. M. Addison, B. D. Alexander, P. Della-Latta, M. Gherna, G. Haase, G. Hall, J. K. John-**

son, W. G. Merz, H. Peltroche-Llacsahuanga, H. Stender, R. A. Venezia, D. Wilson, G. W. Procop, F. Wu, and M. J. Fiandaca. 2008. Multicenter evaluation of the *Candida albicans*/*Candida glabrata* peptide nucleic acid fluorescent in situ hybridization method for simultaneous dual-color identification of *C. albicans* and *C. glabrata* directly from blood culture bottles. *J. Clin. Microbiol.* **46:**50–55.

110. **Shorr, A. F., V. Gupta, X. Sun, R. S. Johannes, J. Spalding, and Y. P. Tabak.** 2009. Burden of early-onset candidemia: analysis of culture-positive bloodstream infections from a large U.S. database. *Crit. Care Med.* **37:**2519–2526; quiz, 2535.
111. **Shorr, A. F., D. R. Lazarus, J. H. Sherner, W. L. Jackson, M. Morrel, V. J. Fraser, and M. H. Kollef.** 2007. Do clinical features allow for accurate prediction of fungal pathogenesis in bloodstream infections? Potential implications of the increasing prevalence of non-*albicans* candidemia. *Crit. Care Med.* **35:**1077–1083.
112. **Sipsas, N. V., R. E. Lewis, J. Tarrand, R. Hachem, K. V. Rolston, I. I. Raad, and D. P. Kontoyiannis.** 2009. Candidemia in patients with hematologic malignancies in the era of new antifungal agents (2001–2007): stable incidence but changing epidemiology of a still frequently lethal infection. *Cancer* **115:**4745–4752.
113. **Slavin, M. A., T. C. Sorrell, D. Marriott, K. A. Thursky, Q. Nguyen, D. H. Ellis, C. O. Morrissey, and S. C. Chen.** 2010. Candidaemia in adult cancer patients: risks for fluconazole-resistant isolates and death. *J. Antimicrob. Chemother.* **65:**1042–1051.
114. **Trick, W. E., S. K. Fridkin, J. R. Edwards, R. A. Hajjeh, and R. P. Gaynes.** 2002. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989-1999. *Clin. Infect. Dis.* **35:**627–630.
115. **Trofa, D., A. Gacser, and J. D. Nosanchuk.** 2008. *Candida parapsilosis*, an emerging fungal pathogen. *Clin. Microbiol. Rev.* **21:**606–625.
116. **Viscoli, C., C. Girmenia, A. Marinus, L. Collette, P. Martino, B. Vandercam, C. Doyen, B. Lebeau, D. Spence, V. Krcmery, B. De Pauw, and F. Meunier.** 1999. Candidemia in cancer patients: a prospective, multicenter surveillance study by the Invasive Fungal Infection Group (IFIG) of the European Organization for Research and Treatment of Cancer (EORTC). *Clin. Infect. Dis.* **28:** 1071–1079.
117. **Walsh, T. J., H. Teppler, G. R. Donowitz, J. A. Maertens, L. R. Baden, A. Dmoszynska, O. A. Cornely, M. R. Bourque, R. J. Lupinacci, C. A. Sable, and B. E. dePauw.** 2004. Caspofungin versus liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia. *N. Engl. J. Med.* **351:** 1391–1402.
118. **Wenzel, R. P.** 1995. Nosocomial candidemia: risk factors and attributable mortality. *Clin. Infect. Dis.* **20:** 1531–1534.
119. **Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel.** 1988. Hospital acquired candidemia: the attributable mortality and excess length of stay. *Arch. Intern. Med.* **148:**2642–2645.
120. **Wilson, D. A., M. J. Joyce, L. S. Hall, L. B. Reller, G. D. Roberts, G. S. Hall, B. D. Alexander, and G. W. Procop.** 2005. Multicenter evaluation of a *Candida albicans* peptide nucleic acid fluorescent in situ hybridization probe for characterization of yeast isolates from blood cultures. *J. Clin. Microbiol.* **43:**2909–2912.
121. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317.
122. **Zaoutis, T. E., J. Argon, J. Chu, J. A. Berlin, T. J. Walsh, and C. Feudtner.** 2005. The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. *Clin. Infect. Dis.* **41:**1232–1239.
123. **Zaoutis, T. E., H. M. Greves, E. Lautenbach, W. B. Bilker, and S. E. Coffin.** 2004. Risk factors for disseminated candidiasis in children with candidemia. *Pediatr. Infect. Dis. J.* **23:**635–641.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch24

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Insights in Antifungal Drug Discovery

FRANÇOISE GAY-ANDRIEU, JARED MAY, DONGMEI LI, NUO SUN, HUI CHEN, RICHARD CALDERONE, AND DEEPU ALEX

What drives the need for new antifungal drugs against *Candida* species? Certainly a lack of efficacy would demand new therapeutics, but is there evidence of this? The answer is complex. Treatment failures against candidiasis are reported which, without question, have negative effects on the quality of life. At the same time, treatment failures against systemic candidiasis may stem from delayed therapy due to the poor sensitivity of existing diagnostic tests, rather than inadequate antifungal activity. So it is perhaps not poor drugs but a lack of sufficient sensitivity and specificity of diagnostic tests that complicates patient management. The complexity of the issue is that candidiasis is not a single disease but, rather, a continuum that reflects many underlying risk factors that may require stratification of patient populations to receive more or less aggressive therapeutic management. We make the assumption that drug development is part of the ongoing commitment of the *Candida* research community to deliver important translational research. After all, candidiasis is a major human disease.

This chapter is divided into two sections. In the first, we focus upon clinical perspectives, especially of global candidiasis. We acknowledge the need to understand that although patient populations and types of candidiasis may be influenced by the economies of developing and developed countries, patients in each setting require the same level of therapeutic quality and care. In the second section, we discuss antifungal drug discovery by offering two different but interacting approaches: traditional, or classical, and genomic. Readers are directed to other chapters in this section of the text that address issues of the current antifungals, such as isolate resistance and its influence on patient management, and pharmacokinetics and pharmacodynamics. These topics are not discussed to any great extent in this chapter. For an excellent review on genomic approaches to drug discovery, see the work of Weig and Brown (122).

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THE INCIDENCE OF GLOBAL CANDIDIASIS

We first address the incidence of global candidiasis. The disease has been reported from every continent. Variation occurs in the types of candidiasis that is reflective mostly of the underlying conditions that predispose patients to this disease. For example, in countries with a very high incidence of human immunodeficiency virus (HIV)/AIDS, oropharyngeal and esophageal forms of candidiasis represent the most common types. Certainly, this correlation is not limited to candidiasis, since cryptococcosis is still a major health problem on the African continent in the same patient population. In contrast, developed countries with effective and available anti-HIV therapies have reduced amounts of oral candidiasis associated with HIV/AIDs patients. However, medical advances to prolong life have resulted in significant morbidity and mortality associated with systemic candidiasis. These are simple correlates but at least point to the variations in candidiasis that most resemble some of the bacterial pathogens in the breadth and severity of disease. Below, we contrast some of the types of candidiasis, with an emphasis on the geographical distribution and its effect on the global burden of these diseases.

Mucosal (Oropharyngeal) Candidiasis and Vulvovaginal Candidiasis

In HIV/AIDS patients especially, mucosal candidiasis, or oropharyngeal candidiasis (OPC), remains one of the most common types of infection throughout the world, but especially in both adult and pediatric age groups in developing countries (7, 38, 48, 72, 80, 88, 93, 130). OPC is the most frequent fungal infection in South African children and adolescents who are HIV positive (95). About 500,000 AIDS orphans exist in South Africa alone, many of whom are HIV positive (7). A 13.8% level of oral candidiasis was noted among 87 pediatric individuals from five orphanages in South Africa; carriage rates in these populations varied from ~37.5 to 80%. These data closely parallel a previous study of Nigerian children that had a 50% carriage rate (48). In Gabon, the prevalence of HIV/AIDS was 27.7% in a hospital population, and among the affected patients, OPC (37%) was the most common of all opportunistic infections

exceeding tuberculosis (14.5%) (80). A study in Tanzania demonstrated that C. *albicans* (84.5%) was the most common species isolated from 250 HIV-infected patients with OPC (38). OPC (37%), salmonellosis (18.2%), and tuberculosis (14.5%) were the most frequently diagnosed opportunistic infections in HIV/AIDS patients in Gabon (80). In China, the two leading causes of oral disease in HIV/AIDS patients are candidiasis (~66% of all patients) and herpes simplex virus (22%) (130). In India, the five leading causes of opportunistic infections in HIV/AIDS patients are candidiasis (88%), tuberculosis (57%), infection with enteropathogenic *Vibrio* (47%), cytomegalovirus infection (45%), and cryptosporidial diarrhea (43%) (20). These data serve to emphasize that in certain settings, candidiasis ranks among the highest in patient populations. Moreover, OPC has not gone away in any location.

The spectrum of mucosal candidiasis also includes vaginal disease. Information on the incidence of vulvovaginal candidiasis (VVC) is incomplete as it is not reportable. However, the estimate is that VVC caused by *Candida* species affects about 70 to 75% of young women of childbearing age (most frequent); 40 to 50% of these individuals will have a recurrence, and 5 to 8% will develop recurrent VVC, defined as four or more episodes per year (26, 108). Therefore, any discussion of frequency and economic impact of candidiasis must include VVC, recurrent VVC, and other types of mucosal infections that are not life threatening.

Systemic Candidiasis

Systemic candidiasis and bloodstream infections (BSI) also qualify as global infectious diseases. An increased incidence of invasive candidiasis (IC), aspergillosis, and zygomycosis has been reported in tertiary care facilities in India (19). Pfaller and Diekema present a very compelling picture of blood-borne candidiasis in the United States (90). Depending upon the total nosocomial infection rate (percent, all microorganisms), valued at 2.5, 5, or 10% and an 8% rate of nosocomial infections caused by *Candida* species, deaths due to these species range from 2,800 to 11,200 per annum. The incidence of candidemia in the United States is highest among the very young (<1 year old) and patients >65 years old. The overall attributable mortality rate due to candidemias ranges from 3.4 to 49% (90). The crude mortality incidences of nosocomial candidiasis in other global sites were 9.2% (Switzerland), 61.8% (France), and 42% (Brazil) (11, 40, 104). There are several studies that correlate data on the incidence of candidemias, length of hospitalization, and mortality with the cost of medical care, which is indicated to be almost $2 billion in health care (69, 90). Globally, bloodstream candidiasis is more frequently caused now by non-*Candida albicans* (NAC) species such as *C. parapsilosis*, *C.tropicalis*, and *C. glabrata*. *C.tropicalis* is a frequent agent of bloodstream infection in both Latin America and the Asia-Pacific region (76). As with OPC, therapeutic intervention in nosocomial disease is becoming increasingly complicated by an increase in drug resistance, notably among NAC species (90).

THE DIFFICULTIES IN TREATING IC ARE NUMEROUS

The preceding and later chapters address many of the problems with patient management. Those problems include the lack of high sensitivity and rapid diagnostic tools, drug resistance and its impact clinically, toxicity, and, last but not least, cost. Equally daunting is the question of when one starts and finishes therapy. Below we address most of these issues.

Diagnostics and IC

A delay in diagnosis of blood-borne candidiasis and treatment results in significantly higher attributable mortality. However, the early diagnosis of IC remains challenging. Positive cultures from nonsterile sites may be related to either colonization or disease, and only the recovery of the organism by culture from a sterile site such as a blood sample can prove an invasive infection (22, 87). Even as blood culture procedures greatly improved during the last decades, with the generalized use of automated broth blood culture systems in most hospitals, sensitivity for the diagnosis of candidemia remains too low (around 50%). Optimally, growth should be detected within 24 to 48 h from patient specimens. In this regard, it is very important to insist on the use of specific yeast blood culture systems. Despite the high cost, this procedure has proved to enhance the sensitivity for fungemia detection and reduce the time to detection (27). In order to improve the diagnostic capability for IC, several nonculture methods have been developed within the last decades, including detection of (1,3)-β-D-glucan and detection of fungal DNA (87). Ideally traditional culture methods could be complemented with one or more non-culture-based microbiological tools to improve diagnosis, sensitivity, and specificity. As detailed elsewhere is this book, progress in this area is still needed. Because of their very high cost, widespread applications of adjunctive procedures are best considered among patients in high-risk wards. Despite the benefit they can offer, these new techniques, especially those based on DNA detection, have their limits, as they do not easily discriminate colonization from IC. Diagnosis based on histopathological samples is often too invasive for critically ill patients, and hence repeated blood culture sampling using specific media remains essential. Of great importance, once a blood culture does become positive for *Candida* species, treatment should be initiated quickly as the window for cure narrows (85, 96).

Antifungal Drugs, Resistance, and Epidemiology: Themes

Antifungal drugs that are currently used in patient treatment of candidiasis include the polyene amphotericin B (AmpB), azoles, and the echinocandins (82). These compounds bind to membrane ergosterol (AmpB) and perturb membrane functions, inhibit ergosterol synthesis (an essential process for growth of fungi) (azoles), or inhibit cell wall β-1,3 glucan synthesis (echinocandins). The standardization of antifungal susceptibility testing especially with regard to *Candida* species has led to active surveillance for resistance (30, 75, 84, 91).

There is by no means complete agreement on the significance of drug resistance and the emergence of candidiasis (see chapter 22). One of the main discoveries of the past few decades in the antifungal field was the development of new azoles, principally fluconazole. With a high efficacy against yeast, especially *Candida albicans*, and low toxicity, this drug was to revolutionize the therapeutic approach to candidiasis and made feasible the concept of prophylaxis (6). At the same time, several years of extensive use of fluconazole may have been responsible for an evolution in invasive fungal infection epidemiology. The main changes reported are a decrease in IC in transplant recipients; a reversal of the pro-

portion of yeast/mold invasive infections; an increase in yeast infections caused by NAC species, especially among patients with hematological malignancies; and, as a potential corollary, an emergence of fungi with reduced susceptibility to fluconazole (17, 37, 60). But the real impact of fluconazole use on these changes is very difficult to assess. Consistent trends are not easy to highlight, as the epidemiology of invasive fungal infections is difficult to study. The first reason for such issues concerns diagnostic difficulties, as described above, which are probably responsible for an underestimation of the rate of invasive fungal infections. To face this problem, postmortem studies are of high interest but limited, as autopsy rates have decreased in many U.S. hospitals due to financial and human resource costs as well as policy constraints. Two studies performed by the same team at two different periods in the same university hospital showed that after a trend to less candidiasis, the prevalence of invasive *Candida* infections then increased again (34, 61). The second reason concerns the heterogeneity of the patient populations in terms of demography, underlying disease, and medical practices, as seen from single-center studies. Multicenter studies are of interest, and several very large studies based on surveillance networks provided a large amount of data showing that the incidence of IC, species distribution, and susceptibility to antifungal agents are highly variable among different patient populations, medical practices, hospitals, regions, and even countries (46, 58, 74, 84, 90).

On the more specific issue of the risk of resistance emerging, a large study was recently published on a 10.5-year surveillance of susceptibility of *Candida* species to fluconazole and voriconazole. With 256,882 and 197,619 isolates from 41 countries analyzed for fluconazole and voriconazole susceptibility, respectively, this study highlights a few important trends. First, it is worth noting for clinicians that overall susceptibility of *Candida* isolates remains high for fluconazole and voriconazole, at 90.2 and 95%, respectively. *C. albicans* more specifically remains highly susceptible to both azoles, but this statement needs to be modified according to the geographical area, as a higher rate of resistance has been observed in North America. This study also reported a slight trend toward an increasing resistance, in some regions, of NAC species like *C. tropicalis* and *C. parapsilosis*. The emergence of the less common and less susceptible, or resistant, NAC species, like *C. krusei*, has been reported, as has the emergence of rare species less known by the clinicians, such as *C. inconspicua* and *C. norvegensis* (92).

Despite the power of this large cohort study, such a global analysis of susceptibility profiles cannot take into account clinical data. Thanks to a systematic record of events taking place within 30 days prior to candidemia, a multicenter study showed that a recent exposure to caspofungin or fluconazole was associated with changes in the epidemiology of the infection (67). For both drugs, preexposure was associated with a decreased prevalence of *C. albicans* in favor of less drug-susceptible species: *C. glabrata* and *C. krusei* after fluconazole and *C. parapsilosis* and, to a lesser extent, *C. glabrata* and *C. krusei* after caspofungin. This study is in favor of a direct impact of antifungal use on the epidemiology of invasive fungal infections. Since the launch of caspofungin, some other studies have already reported the risk of emergence of *C. parapsilosis*, known for its reduced susceptibility to echinocandins (29, 114).

These data demonstrate the absolute necessity to perform prospective epidemiological surveillance, including collection of data on clinical parameters, antifungal preexposure, and treatment. This kind of surveillance should be of high interest at national and international levels in order to provide practical guidelines and also at local levels. Indeed, the knowledge of local epidemiology is the first condition for a more accurate and also a more cost-effective use of antifungals.

How much drug resistance has been reported? In spite of the belief that AmpB is broadly active against *Candida* species, there are data to suggest a less-than-optimal activity against the NAC species *C. rugosa*, *C. krusei*, *C. guilliermondii*, and *C. lusitaniae*, ranging from 5 to 20% of isolates (59). This concern is amplified considerably by the availability of data on resistance to the triazoles (and, to a lesser extent, echinocandins) among *Candida* species as well as *Aspergillus fumigatus* (90, 111). Resistance to both azoles and echinocandins was most prominent among isolates of *C. glabrata*, with the highest rates of resistance to echinocandins (16.7%), fluconazole (16.7%), posaconazole (5.0%), and voriconazole (11.1%) among isolates from the 20- to 39-year age group (89). The resistance rate among *C. glabrata* isolates has been reported to be as high as 18% of North America strains (88). Failure rates of these agents, including voriconazole and caspofungin (an echinocandin), are high—in the range of 60 to 70% in recipients of allogeneic hematopoietic stem cell transplants and 30% of patients with advanced AIDS (8, 114). In the case of *C. parapsilosis* and *C. guilliermondii*, elevated MICs are observed, and in cases of esophagitis, candidemia, and endocarditis, caspofungin resistance has emerged (88, 90, 91, 93, 114). Cross-resistance among *Candida* species to triazoles, defined as resistance that develops during therapy with one triazole that results in resistance to a second triazole that the strain has not seen previously, has been reported. As recently stated (28), therapeutic intervention in candidemia is becoming increasingly complicated by an increase in drug-resistant strains. As resistance varies among species but is much higher in *Candida* NAC species, it is imperative to identify the species of pathogen and also perform drug sensitivity assays.

Risk Assessment

Strategies to minimize blood-borne fungal diseases, including candidiasis, include risk factor assessment, with an increasing interest in antifungal prophylaxis of high-risk patients, and surveillance of drug resistance (30, 75, 84). During the past two decades, we have witnessed an increase in the number and severity of critically ill patients (especially with protracted neutropenia), extensive use of invasive medical devices, wider use of broad-spectrum antimicrobial agents, extensive use of corticosteroids, and, more recently, development of new immunosuppressive agents (T-cell immunosuppressants). All these factors, and others, contribute to the overall risk of invasive fungal infections (22, 37, 119). Moreover, despite new diagnostic tools, the sensitivity of the diagnosis remains too low. Consequently, high-risk populations have to be identified, and among these populations, individual patients with an even higher risk must be recognized. Risk factors are well understood now, as a better understanding of the pathophysiology of invasive fungal diseases has been gained. Nevertheless, the reliable identification of patients at high enough risk for systemic candidiasis that would justify empirical antifungal therapy remains a major clinical challenge.

Colonization of skin and mucosa by *Candida* spp. is the first step to development of infection. *Candida* spp., especially *C. albicans*, may be present as commensals on digestive

and vaginal mucosa in healthy subjects, in balance with other organisms like bacteria. T lymphocytes play a crucial role in the control of the pathogenicity in skin and mucosa, and many factors may disrupt this balance: disequilibrium of the bacterial flora due to antibiotic treatment, mucosal damage, lack of control by $CD4^+$ lymphocytes (as occurs during HIV/AIDS or after treatment with anti-CD52 monoclonal antibody [alemtuzumab] that is used in the treatment of chronic lymphocytic leukemia), high levels of glucose due to diabetes, or estrogen treatment (116). It is important to note that the evolution from colonization to disease is a continuum; consequently, to distinguish the two remains a difficult challenge. Invasion is also highly facilitated in the case of epithelium damage (surgery, catheters, etc.). During invasion, macrophages and neutrophils are recruited to the site of infection for phagocytosis and killing of *Candida*, while contributing to cytokine production. Severe invasive *Candida* infections may occur in patients with abnormal neutrophil recruitment, neutrophil dysfunction, or severe neutropenia. The most common acquired cause of defective neutrophil recruitment is treatment with glucocorticosteroids or other immunosuppressive agents (116). All of these risk factors are nonspecific, but their recognition allows targeting of high-risk populations. The differential role of T lymphocytes and macrophages during the course of *Candida* infection explains why as macrophage function is preserved during HIV infection, patients suffering from HIV/AIDS are exposed to superficial candidiasis (OPC) rather than disseminated visceral candidiasis. Patients at risk for IC are those with a recent history of neutropenia, allogeneic stem cell transplant, prolonged use of corticosteroids, treatment with other recognized T-cell immunosuppressants, or inherited severe immunodeficiency (22). Among these populations, some patients manifest several risk factors like broad-spectrum antibacterial drugs, previous colonization, indwelling catheters, total parenteral nutrition, prior surgery (especially gastrointestinal), renal failure and/or hemodialysis, and mechanical ventilation. Other factors have also been shown to increase the risk of acquiring candidemia: age (neonates/premature infants and patients greater than 70 year of age), malnutrition, prolonged hospital stay (especially in an intensive care unit [ICU]), and, of course, severity of underlying disease, which is linked to many of the previous factors (90, 116). Patients with several risk factors should be targeted for more specific diagnostic tests and considered for prophylaxis or for empirical treatment if IC is suspected but not proven (85).

Incidence and Health Care Cost of Fungal Disease

Fungal infections have risen sharply over the past few decades. From 1980 to 1997, invasive mycotic disease increased from the tenth most common cause of deaths due to infectious disease to the seventh. The five leading mycoses during this time were candidiasis, including IC, invasive aspergillosis (IA), histoplasmosis, cryptococcosis, and coccidioidomycosis (90). The increase was at least partially due to the rapid emergence of the AIDS pandemic, but other comorbidities such as immunosuppressive cancer chemotherapy and immunosuppression following bone marrow transplantation, surgery, ventilators, and indwelling central-line catheters were responsible, especially in patients that developed IC and IA.

Candida species rank among the top four causes of bacterial/fungal nosocomial infectious diseases in humans, and *A. fumigatus* is the most deadly and frequent mould infection of humans. Pfaller and Diekema followed the incidence of IC and IA from 1996 to 2003 (90). Their data are similar to those of other groups in that the incidence of IC has remained steady over the past decade, while IA may be decreasing but still remains a common and devastating disease.

There are several studies that correlate data on the incidence of candidemia, length of hospitalization, and mortality with the cost of medical cure. Miller et al. compared patient data from two sites in the United States (69). While there was significant heterogeneity, the median total hospital charge was $44,696 to $77,534 per patient. Candidemia thus carries with it a significant financial burden, estimated at $1.7 billion, and overall, the cost burden of fungal infections in the United States is estimated to be $2.6 billion per year, or about 0.24% of the total U.S. health expenditures (125).

Expense to patients, hospitals, and even providers is associated with a number of factors, not the least of which is length of stay (LOS) in a hospital when an infection develops (131). For example, the LOS reported for candidiasis depended upon whether the patient was in an ICU (12.7 days) or a non-ICU setting (15.5 days) (81). The reason for the increased LOS points to inadequate diagnostic assays. Bacteremia is detected much earlier and at a higher frequency than candidemia, since diagnostic assays for candidiasis lack sufficient sensitivity, precluding early diagnosis. In ICUs, patients at high risk for candidiasis can be identified using predictive outcomes, including APACHE scores and other parameters (57). Data have shown that there is a narrow window of opportunity for effective therapy once a blood culture becomes positive. Since LOS is longer for candidiasis patients, this obviously results in a higher expense for treatment and cure.

Cost issues are becoming a critical point in the treatment of systemic fungal infections since the introduction of much more expensive new generations of antifungals. The expenditure for antifungal drugs has risen sharply over the last few years and tends now to be as high as those for antibiotics in some hospitals, especially in the care of high-risk patients (see above). A study based on data before 1996 estimated the cost of an episode of care for candidemia in the United States between $34,123 (Medicare) and $44,536 (private insurance). At that time the major cost associated with candidemia was that of increased hospital stays, which accounted for 85 to 90% of the total cost. All patients received AmpB (71%) or fluconazole (29%), with a cost per treatment of, respectively, $2,471 or $2,874 for Medicare patients (99). In the following years, the newly developed lipid-associated AmpB was shown to be a promising option for the care of invasive fungal infections, with fewer side effects and decreased nephrotoxicity than conventional AmpB (43, 97). But because of the much higher acquisition cost of these new molecules (see http://www.doctorfungus.org/thedrugs/cost1.php, *2006 Drug Topics Redbook*), many questions have arisen concerning the medical, but also economic, benefit of these new treatments. Many studies were conducted aiming to assess the more cost-effective therapeutic option in different clinical situations. For example, in a pharmacoeconomic analysis of liposomal AmpB versus conventional AmpB in the empirical treatment of persistently febrile neutropenic patients, Cagnoni et al. showed that the mean cost of medication was significantly greater for patients treated with liposomal AmpB because of higher dosing regimens and a higher drug average wholesale price (AWP). But when the costs of the drugs were not included in the analysis, hospital costs were lower for patients treated

with liposomal AmpB, probably because of the costs induced by nephrotoxicity, which appeared to be more frequent in patients treated with conventional AmpB. Based on these results, it has been estimated that the acquisition cost of liposomal AmpB would have been 50% of the AWP at the time of the study to be cost-effective (15). But this conclusion has to be pondered, as the cost of a drug varies from country to country, and varies also among individual institutions according to contract prices. Studies that analyze the cost of the drug should not concentrate only on the acquisition cost of the medication but need to integrate the cost of the illness (117). As explained above, invasive fungal infections are difficult to diagnose and affect critically ill patients, sometimes with poor prognosis. Thus, the global cost of fungal invasive disease may be very high. Increased LOS raises not just direct medical costs (room and board, pharmacy [excluding antifungals], laboratory services, radiology, transfusions, and nutrition) but also indirect costs (e.g., absenteeism) and intangible costs (e.g., quality of life) (15, 77, 117). The choice between the use of conventional and new formulations of AmpB is not the only dilemma faced by the clinician. As the armamentarium of antifungals increases, other difficult choices have to be made on a daily basis. Facing a case of IC, the clinician can use fluconazole, echinocandins, expanded-spectrum azoles, or new formulations of AmpB (85). Pharmacoeconomic considerations need to be part of the reflection, but a single conclusion cannot be drawn because of the multiplicity of clinical situations. Many factors are involved in choosing a therapy, and among the most important are the presence or absence of neutropenia, the severity of the underlying illness, preexposure to antifungals, and the risk for the patient to develop irreversible nephrotoxicity. Guidelines are published and decision analysis models are available to develop a more rational use of antifungal drugs. Most of these models are of invasive fungal infections in industrialized countries. However, such approaches could also be implemented in developing countries. A recent study was conducted in Cambodia (where cryptococcal meningitis is an important cause of mortality among AIDS patients) to assess the cost-effectiveness of primary prophylaxis with fluconazole. Unlike previous results obtained in industrialized countries, where the incidence of the disease is not the same, this study showed that in an area where cryptococcosis and AIDS are highly endemic, prophylaxis is a cost-effective strategy to prevent AIDS-associated cryptococcosis (68). However, the acquisition cost of antifungals remains a crucial concern for developing countries. Some initiatives have been developed to make existing medicines available for the poorest. One example is the Diflucan Partnership Program between Pfizer Inc. and the South African Ministry of Health to distribute free Diflucan (fluconazole) in a context of a high AIDS prevalence (123). In addition to the context of candidiasis, one must not forget that antifungals are essential to treat endemic mycoses worldwide. Until now, the recommendations for the first-line treatment of these endemic mycoses were conventional molecules, mainly AmpB, itraconazole, and ketoconazole. But the clinical outcome is not always optimal, and some of these diseases, for example, chromoblastomycosis or eumycetoma, respond poorly to medical treatment (66). Thus, for these kinds of infections, it is tempting to consider that more recent antifungals, such as terbinafine, voriconazole, and posaconazole, could be useful. In this regard, some encouraging results have been published (1, 9). However, the medical community needs to be aware that the acquisition price of these drugs will prevent their use in developing countries by patients who cannot afford the cost of much cheaper drugs, like generic forms of AmpB, itraconazole, and ketoconazole.

APPROACHES TO NEW ANTIFUNGAL DRUG DISCOVERY

It is quite clear that progress towards the identification of new classes of antifungals has not been made. Drugs that are currently used in the clinic target ergosterol synthesis, membrane ergosterol, or β-1,3 glucan synthesis, representing about 0.0004% of the entire genome of *C. albicans*. New drugs are remodeled triazoles or echinocandins. The same lack of discovery is witnessed in antibacterial drug development, so that terms such as "bad bugs-no drugs" now reflect the nature of pathogens resistant to nearly all available antibacterial drugs on hand to treat these infections (10, 47a).

The reasons for a lack of new classes of drugs and the use of remodeled ones are numerous and include the costs of research and development, loss of patent rights, reduced capital venture, and the paradoxical concept that drugs which cure in a short time are in general not as lucrative as those that require use for extended periods. Nevertheless, as a response to new drug shortage, the Infectious Disease Society of America and the European counterparts are planning new assaults specifically on drug-resistant organisms.

In this section, we have organized drug discovery into two approaches, each of which begins with a different initial focus. The first of these approaches might be referred to as classical or traditional, whereby a putative new antifungal is identified from large compound libraries that are used in screens against an array of species that appropriately should include *Saccharomyces cerevisiae*. The reason for choosing this nonpathogen are explained below. The second approach seeks a target that is broadly represented in numerous fungal pathogens, important for growth or even virulence of fungi, and not found in human cells. This approach relies upon genomic base searches of fungal pathogens and, when possible, functional annotation. If the other features mentioned above are indicated but without functional annotation, then putative targets require verification of their importance to growth or for disease to occur. The so-called "antivirulence" drugs (18) have gained a conceptual foothold in the antibacterial drug discovery paradigm but seem to be mostly ignored in the fungal literature. Below, both models of drug discovery are described in some detail (Fig. 1). Our descriptions do not mean to imply that these approaches cannot be integrated. In fact, the examples given below indicate such. Thus, compounds of interest can be used in screens of genetic mutants to define a mode of action (MOA) of a new antimicrobial that displays in vitro activity to target organisms. Genetic screens utilize the power of several *S. cerevisiae* mutant libraries defined below. As for similar libraries in human-pathogenic fungi, methods have evolved that utilize either conditional or heterozygotic mutants that display a phenotype referred to as haploinsufficiency (HI). Similar mutant libraries exist in *S. cerevisiae* but usually with the advantage of total or near-total genome coverage.

Classical/Traditional Approach (Drug Based)

The two initial requisites for the classical approach are a suitable compound library and pathogens (or a target) of interest to use in screens of the compound library. Of these, finding a compound library can be problematic. There are two possibilities. We have been fortunate to collaborate

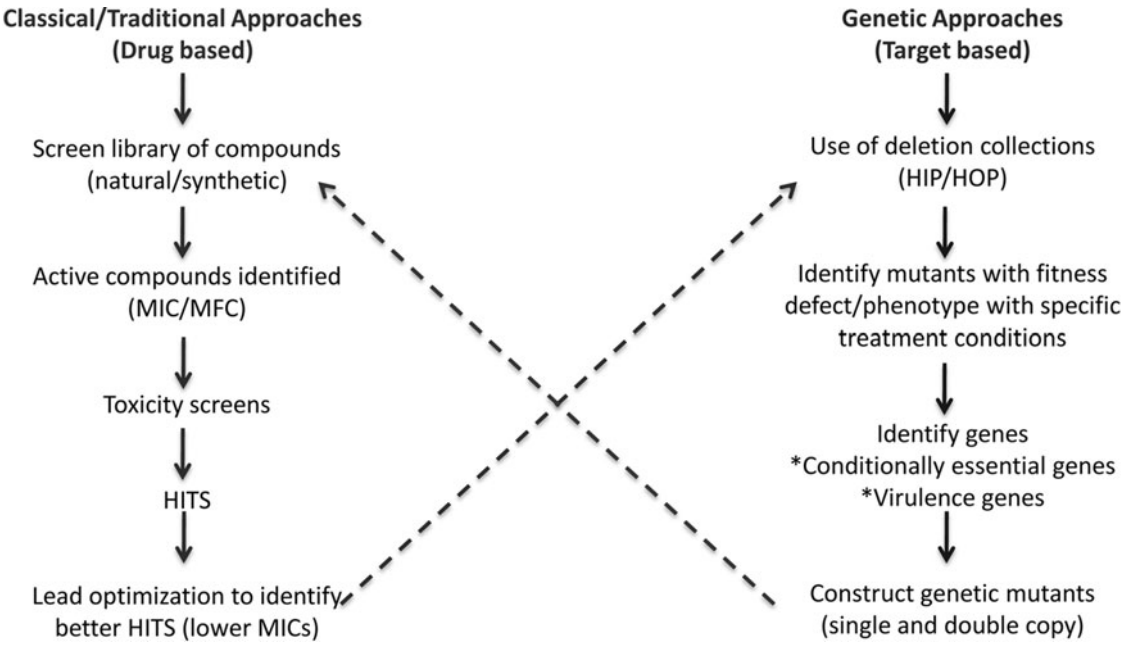

FIGURE 1 The two pathways to antifungal drug discovery. The traditional, or classical, pathway starts with compound libraries that screen a panel of fungal species. Compounds are selected as fungicidal or fungistatic, optimized for activity, and tested for in vitro toxicity, and the best lead optimized compounds are selected. Genomic approaches start with deletion sets of mutants that can provide full coverage of the genome or partial sets (as for example, growth essential genes). The deletion sets are referred to as HIP or HOP (from HI profiling or homozygous deletion profiling, respectively), reflecting the nature of the set of mutants. Mutants of genes of interest are screened against compound libraries to identify hit compounds (left-pointing diagonal arrow). Or, compounds identified by classical methods are used against sets of mutants to identify the genetic target(s) (right-pointing diagonal arrow). MFC, minimal fungicidal concentration. 10.1128/9781555817176.ch24f1

with a medicinal chemist who provides ample numbers of scaffolds (families) of compounds. Active scaffolds are modified further to optimize a "hit" compound. Also, the National Institutes of Health in the United States currently provides a list of centers (Molecular Libraries Program Centers Network) that are available for many types of assay developments, including those that assess anti-infective activity. The assays are robust and can consist of libraries of ~200,000 to 300,000 compounds for high-throughput screens. To view the site and capabilities of each, see http://mli.nih.gov/mli/mlpcn/. Funding with a specific center is possible once collaborations or potential collaborations are defined. The Helmholtz Center for Infection Research in Germany is one of three centers that will soon begin a coordinated effort for academic screening platforms in Europe called EU-OPENSCREEN (see http://www.helmholtz-hzi.de/en/).

Target-based screens (cell enzymes, for instance) have in general fallen in popularity, whereas cell-based phenotypic screens are preferred for several reasons, especially since "hit" compounds by their inhibitory activity must be also permeable to the test organism(s). In the case of target screens, permeability to the compound would have to be determined eventually. Also, cell-based screens are rather straightforward and require only the selection of broad pathogen representation, including *C. albicans* and NAC species. Certainly among others, *A. fumigatus*, *Cryptococcus neoformans*, dimorphic and endemic fungal pathogens, and rare pathogens such as *Fusarium* species and others should be included. The problems encountered are those pathogens for which standardized susceptibility testing methods are not established, and these include most of the above except for a few of the *Candida* species.

Compound Libraries

There are two types of compound libraries: synthetic and natural. Natural libraries are structurally diverse and have been evolutionarily selected (12). Historically, most drugs evolved from natural compounds or derivatives of natural products. With the advent of high throughput screening methods, there has been a gradual decline in research relating to screening of natural compounds over the past decade. However, the use of newer technologies for natural compound screening and the tapping of new biological resources provide exciting new opportunities for natural product drug discovery (64). Synthetic libraries use combinatorial chemistry to synthesize thousands to millions of ligands, which provides endless possibilities of structures. Within synthetic libraries, there are three main types as described by Drewry and Young (24). They are focused, targeted, and primary screening libraries. A focused library is based on a lead molecule and is designed for a known molecular target, while a targeted library is designed to target a particular class of proteins (e.g., kinases). Finally, a primary screening library is structurally diverse and used to screen against targets with little known information to search for a new lead compound

(24). Once an active compound is identified, it will undergo lead optimization, in which modifications are made to the compound but the core scaffold remains unchanged. The slightly modified compounds will then be used for susceptibility testing to determine if the activity has increased, decreased, or not changed. The goal of lead optimization is to find a compound with the lowest possible MIC that inhibits a panel of fungal pathogens. In the process of selecting hits based on MIC measurements, it is also important to determine if the compounds are fungicidal or fungistatic. In general, the former types of compounds are of greater interest, as recurrent infection may be more likely after the completion of therapy with fungistatic agents. Fungicidal compounds are particularly useful in immunocompromised patients since their immune systems may not be able to eradicate the fungal pathogen (16). Of the commonly used antifungals, AmpB and the echinocandins exhibit fungicidal activity, while the triazoles and flucytosine are fungistatic drugs (62).

Toxicity Studies

Potential hit compounds from the screening process must go through a preliminary stage of toxicity assessment. The toxicity tests are done in vitro with standard mammalian cell lines to determine if the active compounds are toxic. More elaborate toxicity testing and in vivo studies in animal models may be reserved for later stages of the drug development process. The cell line is selected based on the assumption that organs, like the liver and kidneys, that are involved in drug metabolism and excretion may be most exposed to the damaging effects of the compound. In addition, endothelial cell lines may also be tested to look for toxicity in blood vessels after systemic administration or absorption. Toxicity tests measure viability on prolonged exposure to various concentrations of compounds. The neutral red uptake assay for cell viability is one such test. This assay measures cell viability and is one of the most commonly used toxicity tests (100). Viable cells bind and incorporate the dye which concentrates in cell lysosomes. Upon extraction with acidified ethanol, dye content is measured by spectrophotometry. Another photometric test, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (71), is based on the ability of mitochondrial reductase (which is active only in living cells) to reduce MTT to a purple formazan dye and the results are read by spectrophotometry. Other assays to test drug toxicity include trypan blue and lactate dehydrogenase release (118). It is also important to use at least two separate toxicity tests to avoid overestimation of toxicity if the compound targets organelles that may be involved in measuring viability in one of these tests. Based on results from MIC measurements, lead optimization, and toxicity testing, a structure-activity relationship (SAR) can be charted between the organism and active compounds of the same structural scaffold. This is instrumental in the identification and evaluation of the target site.

MOA Studies

The determination of MOA of novel compounds is probably the most challenging aspect of drug discovery. Once an active compound has been identified, it is important to ascertain if the MOA may be related to pathways inhibited by already existing antifungals. To validate that the new hit compounds are active against targets other than those already used in the clinic, one approach is to screen a number of azole- and echinocandin-resistant *C. albicans* and *C. glabrata* strains that either have target point mutations or overexpress their target genes. If these mutants are in fact sensitive to the hit compound, then the MOA is very likely different for these compounds versus current drugs.

Another method to gain information of an MOA is to measure the effect of the hit compound on macromolecular synthesis. In this case, cells are pulse-labeled with radiolabeled precursors (glucose, leucine, uridine, thymidine, etc.), and incorporation is observed (56). If incorporation of a precursor is inhibited, then the compound is most likely involved in inhibition of that pathway (e.g., β-1,3 glucan synthesis).

As mentioned above, compounds preferably should also be inhibitory to *S. cerevisiae*. This is important since it provides a way in which the cell target(s), and hence MOA, can be identified, using mutant libraries grown in the presence of hit compounds. There are three types of yeast libraries, each consisting of ~4,700 to ≥6,000 mutants representing the entire genome of yeast constructed in a diploid background, including (i) null strains lacking both copies of genes that may be hypersensitive or resistant; (ii) strains that are hemizygotic and display an HI (discussed below) that reveals sensitivity to compounds; and (iii) strains that overexpress targets; those strains that exhibit a resistance phenotype would be of interest. Each of these libraries is available through the European *Saccharomyces cerevisiae* Archive for functional analysis (EUROSCARF) (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html). Every mutant in the library can be screened in 96-well microtiter plates or agar plate assays with the hit compound incorporated. The mutants that are sensitive or resistant to the compound are compiled, and the genes are grouped into functional categories to identify a target or pathway (or both) using algorithms such as Funspec (http://funspec.med.utoronto.ca/) or Gene Ontology (http://www.geneontology.org/). Screening of mutants can also be achieved in batch cultures. Each strain is specifically bar-coded, which enables identification of sensitive (absence) or resistant (survival) strains after treated cultures are compared to untreated cultures. In a study by Arita et al. (3), the nonessential haploid gene knockout strains of *S. cerevisiae* were screened with nickel sulfate. A total of 149 sensitive strains were found in the screen (3.1% of all strains). The genes were then clustered on the basis of functional categories. As expected for nickel sulfate treatment, mutations involved in the homeostasis of metal ions were represented among hypersensitive strains (3). The authors also constructed a protein interactome map to chart out potential interactions (protein-protein, DNA-protein, etc.) that may be important for drug inhibition.

While the phenotype of strains would be seemingly easily identified by the use of one or more of such libraries, there are inherent problems with each library. For example, HI assays may be insufficient to detect hypersensitivity if more than 50% of a gene product is required for hypersensitivity. Null mutants in growth-essential genes are not among the pool for screens. However, nonessential genes of sensitive null mutants identify genes that buffer the drug target or target pathway and are therefore required for growth in the presence of a target compound (107). Also, in *S. cerevisiae*, 15% of strains that over express genes exhibit toxicity to that gene product, which is manifested as retarded growth (109).

Comparative Expression Profiling

Genes that are up- or downregulated on exposure to a compound may be involved in the target pathway. In order to

study this, a microarray analysis is done between an untreated strain and a strain exposed to suboptimal levels of the compound. The resulting change in expression profiles of genes can be grouped to identify a potential target(s) or pathways (65). It is also possible to look for changes over time and concentration gradients that may then give a better picture of transcriptional changes that are important for activity of the compound.

A combination of approaches is vital to gain information about pathways that may be involved in the anti-infective activity of the compound. Genes that show consistent changes in multiple screens (as shown by the areas of overlap in Fig. 2) along with macromolecular synthesis will most likely be related to the target gene(s) of the compound.

Genomic Approaches (Target Based)

For pathogenic fungi like *C. albicans*, a number of efforts have resulted in the development of mutant libraries that have been exploited in proof-of-principle experiments to assess sensitivity to compounds or identify other phenotypes such as those genes required for morphogenesis. Importantly, genomic sequence data for several *Candida* species and other fungi allow the identification of potential targets (122). In this regard, genomic approaches with potential for new antifungal drug discovery initially were focused on developing libraries of *C. albicans* mutants in essential growth genes (33, 103). To do this, a genetic comparison of *C. albicans* genes sharing homology to essential genes of *S. cerevisiae* (32) was done that revealed 864 *C. albicans* genes with significant homology. Next came the arduous task of making *C. albicans* conditional mutants, and of this pool of genes, 823 mutants were constructed. To construct specific gene deletion mutants, one allele was replaced using a PCR-generated disruption cassette containing a selectable marker. Two distinct bar codes (up and down tags) were flanked by two primer pairs that permitted PCR amplification of bar codes and, therefore, identification of each mutant. For the second allele, bar-coded strains were then PCR amplified to introduce the replaceable tetracycline promoter to generate conditional mutants. This functional genome approach was referred to as GRACE (gene replacement and conditional expression). In this way, 567 essential genes of *C. albicans* were identified (103). Of all the *S. cerevisiae* essential gene sets, only 61% were found in *C. albicans*. Of importance, whole-cell assays with conditionally regulated strains enable drug screening of any target that is among the essential gene set of strains (103).

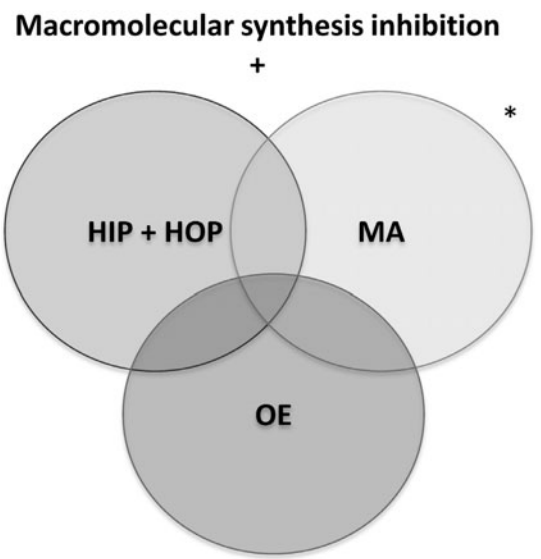

FIGURE 2 Integration of approaches, including macromolecular synthesis inhibition, microarray (MA), deletion sets (HI profiling [HIP] and homozygous deletion profiling [HOP]), and overexpression screens (OE) to identify targets of new drugs. The genes that belong to common areas in the diagram indicate the probable target(s) of a specific compound. The asterisk indicates that genetic mutants were identified from various screens. 10.1128/9781555817176.ch24f2

Following this approach, Xu et al. (126) described the construction of a library of 2,868 heterozygous deletion mutants (~45% of the *C. albicans* genome). The concept for constructing this mutant set was similar to the above except that the mutants were not conditionally expressed. All strains were then used in screens of 35 known or novel compounds to identify chemically induced HI, an assay also referred to as the *C. albicans* fitness test, in which sensitive or resistant strains can be predicted by relative amounts of their specific bar codes in batch cultures of mutants (126). The authors conclude that the *C. albicans* fitness test facilitates a pathogen-focused approach to new antifungal discovery. This application had been applied initially to *S. cerevisiae*, as mentioned above, and resulted in the identification of novel cell interactions as well as the chemical core structure of inhibitors of an *ERG24* heterozygous yeast deletion set (4, 5, 33).

An extension of the principle of fitness has been demonstrated in studies to define the MOA of guanosine 5′-monophosphate synthase inhibitors in *C. albicans* (GMPS, encoded by the *C. albicans GUA1*) and *A. fumigatus* (102). Of approximately 2,868 heterozygous deletion strains, 1 corresponding to the *gua1* null mutation was hypersensitive to the test compound (ECC1385), which additionally inhibited the in vitro activity of the *C. albicans* GMPS. Also, fitness profiling has been used to identify the active components (parnafungins) from natural products of a *Fusarium* culture and show that the target was mRNA processing (49, 86).

More recently, a library of 3,633 tagged and heterozygous transposon mutants of *C. albicans* were screened for HI under varied growth conditions, including chemically diverse compounds (79). For instance, the target of brefelden (an inhibitor of protein transport from the endoplasmic reticulum to the Golgi complex) was identified as Sec7p, while a *C. albicans*-specific target (Tfp1p) was identified as the target for a new anti-*Candida* compound (79). Thus, proof of principle indicates the utility of tagged, mutant libraries, even though only partial ones are available at this time, for screening of potential antifungal compounds. The examples given above reveal the power of mutant libraries in identifying novel targets. These assays require intense labor and a high cost to develop. Yet, the investigator needs to be aware. Toxicity issues and even a lack of in vivo efficacy can still prevent the compound from advancing to therapeutic potential (102). The latter statement points out the risks that are heavily burdensome to drug discovery.

The *Saccharomyces* deletion collections have been used extensively to evaluate responses to a variety of environmental and chemical stressors. Data are available from screening the collections (approximately 6,000 strains) with

more than 700 different treatment conditions (44). It is now possible to have fitness maps (of all the mutants in the collection) to a wide variety of conditions. This can be highly beneficial in identifying possible MOAs of new compounds by comparing fitness profiles. This concept is called coinhibition. The coinhibition profile of a new active compound to a known treatment will determine the extent to which the MOAs are related. The higher the coinhibition score, the closer the association between target pathways. Coinhibition profiles can also be compared among compounds that belong to the same structural scaffold to look for SARs that may not be evident by MIC screening alone. Similarly, the functional closeness of two genes can be analyzed by comparing fitness profiles of their mutants against many tested conditions. This is called cofitness. Cofitness scores can be used to identify genes that may be related to the target pathway of the treatment compound, understand functional roles of related genes under specific conditions, and identify essential complexes. With the vast amount of data available from these screens, it is possible to build interaction profiles between already existing cofitness/coinhibition profiles and that of the test compound (Fig. 3). This may help in the identification of drug target pathways, associated pathways, related treatments, and conditional relationships between compounds and genes that cannot be determined by evaluating individual fitness profiles.

NEW DRUGS IN DEVELOPMENT AGAINST FUNGAL PATHOGENS

The discovery and development of antifungal drugs have constituted a long and interesting journey, beginning with the FDA approval of the polyene AmpB in the 1950s. The number of therapeutic agents against fungal pathogens witnessed a dramatic leap with the development of azole drugs in the 1980s and the introduction of liposomal amphotericin formulations in the 1990s. Echinocandins, the newest addition to the arsenal of antifungals, were successfully added to the therapeutic choices for invasive infections in 2001. Despite the fact that three classes of drugs (polyenes, azoles, and echinocandins) are currently available for treatment of invasive fungal infections, for reasons explained above, new antifungal agents and classes are the need of the hour. With this in mind, drugs are being developed to target new pathways or work on existing targets. New compounds that belong to already existing antifungal classes look for

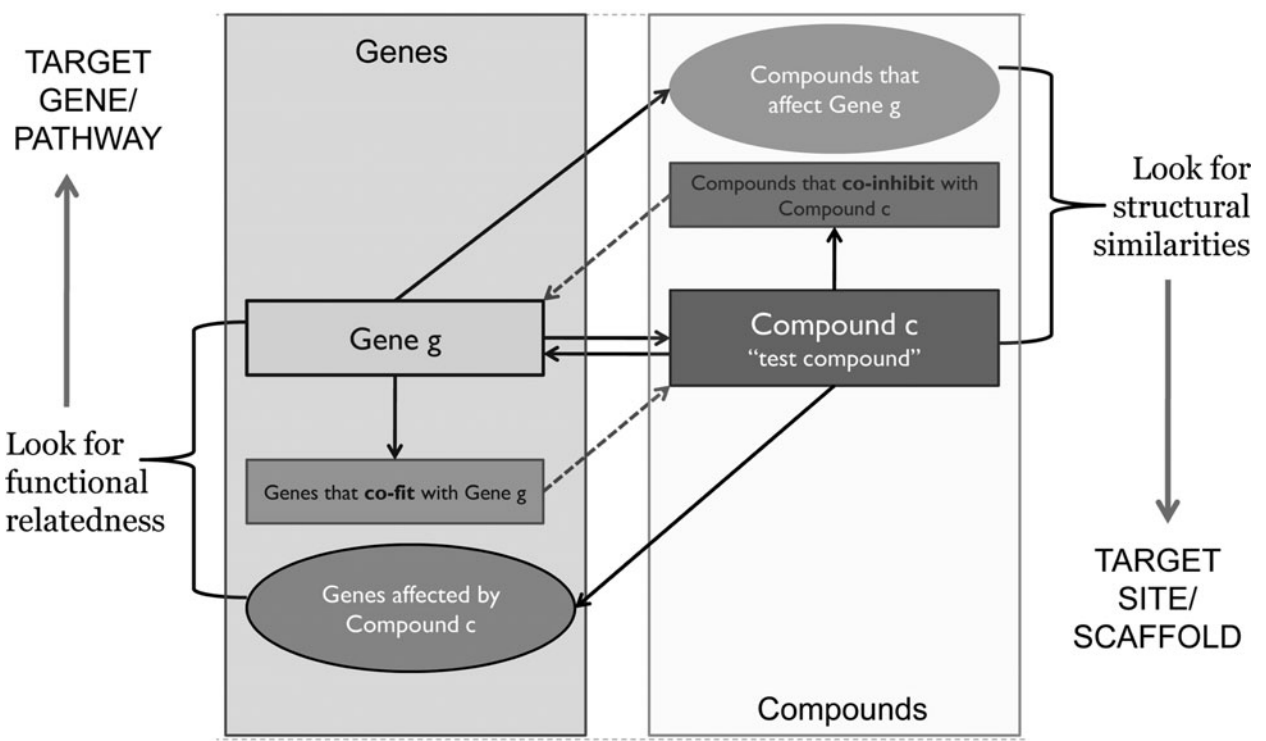

FIGURE 3 The use of interaction profiles in determining active scaffolds and drug targets. A specific phenotypic interaction between gene "g" and compound "c" is used as the basis for this interaction profile. Compounds that have high coinhibition scores with compound c and other compounds that have the same phenotypic interaction with gene g are compared for structural relatedness to identify active scaffolds or possible target sites. Similarly, genes that have high cofitness scores with gene g and other genes that have the same phenotypic interaction with compound c are compared to look for functional relatedness to identify target genes or pathways. Also, the interaction between compounds with high coinhibition scores with compound c and gene g is evaluated. Similarly, the interaction between genes that have high cofitness scores with gene g and compound c can be determined (dotted arrows). 10.1128/9781555817176.ch24f3

structural modifications that decrease toxicity and increase bioavailability and effectiveness.

New Azoles

A new triazole agent, albaconazole, is currently being developed for use against *Candida*, *Cryptococcus*, and *Aspergillus* spp. (82, 110). Isavuconazole, or BAL4815, also a modified triazole, is in clinical development (phase III) for use in treating aspergillosis and zygomycosis and has been shown to be more effective than voriconazole and itraconazole against *Candida* spp. (25, 106). SARs in the development of azole derivatives have yielded new data on compounds with MICs in the range of nanograms/milliliter (35). These compounds act by optimizing the interaction of molecules to the active site of CYP51 in *Candida albicans*. Molecular modeling methods combined with the study of active derivatives are now being used to chart out interactions between the active site and potential compounds with better antifungal profiles (36). Although many such molecules or derivatives of triazoles are now being investigated for in vitro activity, there is a scarcity of data showing definitive advantages compared to existing drugs in clinical trials (82).

New Polyenes

The use of liposomal formulations and lipid complexes of AmpB significantly reduces renal toxicity. A new cochleate formulation has shown efficacy in animal models of candidiasis and aspergillosis (21, 82, 105, 129). The use of lipid nanoparticles, another method to deliver AmpB, shows promise because it significantly reduces toxicity and increases the antifungal activity (50, 82). A liposomal formulation of nystatin is also being investigated (2, 82). A novel polyene, SPK-843, was shown to have better efficacy than established polyenes in a murine model of pulmonary aspergillosis (25, 52). Recent studies investigating the use of aerosolized liposomal AmpB and aerosolized voriconazole for the treatment of invasive pulmonary aspergillosis also look promising (101, 113).

New Echinocandins

The currently used echinocandins (caspofungin, micafungin, and anidulafungin) are still relatively new, and studies relating to their in vivo efficacy are ongoing (82). Aminocandin (IP960/HMR3270), a new echinocandin, is under clinical development for use against *Candida* spp. and filamentous fungi (31, 120, 121).

Other Agents in Development

T-2307 is a novel arylamide that is active against *Candida*, *Cryptococcus*, and *Aspergillus* spp. It is hypothesized that the MOA may be related to mitochondrial functions specific to fungal cells (70, 127). Icofungipen (PLD-118) is a beta amino acid that is active against *Candida* spp. by inhibiting isoleucyl-tRNA synthetase in protein biosynthesis. In vivo activity of this compound against azole-resistant strains has also been demonstrated in murine models (41). Nikkomycin Z, a compound that competitively inhibits chitin synthase in the fungal cell wall, was first identified in the 1970s (14). Both in vitro and in vivo activities have been shown in models of coccidioidomycosis and blastomycosis (42). Recently, there has been renewed interest in chitin synthase inhibitors, and many natural compounds belonging to the same class have been identified (54, 55, 128). Sordarins work by inhibition of elongation factor 2 in protein synthesis (23, 51). Sordarin derivatives were shown to have efficacy in various animal models and are active against *Candida albicans* (39, 53, 78). Miltefosine is an alkyl phosphocholine that acts by the inhibition of lysophospholipases. It has broad-spectrum antifungal activity and is active in vivo in a murine model of disseminated cryptococcosis (124). MGCD290, a Hos2 fungal histone deacetylase inhibitor, has been shown to work synergistically with azoles against the majority of clinical isolates, including azole-resistant yeasts and molds (94). FK506 and cyclosporine are calcineurin inhibitors that are fungicidal and show synergy with fluconazole by preventing the growth of azole-resistant strains in biofilms (115). 75-4590, a pyridobenzimidazole, is active against *Candida* spp. by inhibition of β-1,6 glucan synthesis in the fungal cell wall (56). Analysis of resistant genetic mutants of *Saccharomyces cerevisiae* showed that the target was Kre6p, a β-1,6 glucan synthase (73). There is evidence that monoclonal antibodies that inhibit heat shock protein 90 when used in combination with AmpB or caspofungin leads to significant clinical improvement in cases of IC (45, 83). Studies have shown that Als1p, a cell surface adherence protein, is effective in protecting mice against disseminated candidiasis by enhancing cell-mediated immunity (47, 112). β glucan conjugates have been shown to confer protection in murine models and are also being investigated as potential vaccine candidates for the future (13).

SUMMARY: THE MYTHS AND SELF-IMPOSED LAND MINES OF ANTIFUNGAL DRUG DISCOVERY

The routes to antifungal drug discovery (like any drug) are fraught with speed bumps, one of which is shared toxicity of lead compounds to the fungus and mammals. This fact almost always prevents movement of potential compounds to the user list of therapies. Obviously, there are other reasons, such as good in vitro but minimal in vivo efficacy. However, additional conceptual problems create stops in drug discovery. For example, the emphasis on the potential of a specific compound or family of compounds is that they must be broad in specificity and directed against essential gene products. We raise two counterpoints to these specifications. First, why is broad specificity required? Compounds that act only on *Candida* species or on *C. neoformans* would be efficacious, marketable, and useful in the treatment of by far the leading types of fungal diseases, including oropharyngeal, vaginal, or recurrent vaginal infections as well as cryptococcal infections in HIV/AIDS patients, the latter being a major problem in developing countries. For these drugs, it does not really matter if the compound does or does not kill *A. fumigatus*. Our hypothesis is that in developing countries the major fungal diseases are those in HIV/AIDS patients that include the above-mentioned pathogens. Insistence that new compounds have to kill every fungal pathogen is certainly not the case with the echinocandins, for which *C. neoformans* lacks the drug target. It is now an approach of antibacterial drug discovery to develop drugs against specific types of resistant bacteria. We advocate for targeting of specific patient populations. And, interestingly, in the section on new drugs above, it appears that some are targeted against specific combinations of pathogens. The numbers on incidence and costs to treat fungal infections readily suggest that a market exists.

Second, why do drugs have to be developed solely to essential gene products? There are sufficient examples of broad functions and fungus-specific targets that are critical to virulence, one being the two-component histidine kinases

(HKs). Antibacterial compounds that target HKs that regulate biofilm formation in *Staphylococcus epidermidis* (98) are sought, one of many antivirulence therapies, or therapies directed at virulence factors or regulators of virulence (18). Our data and those of many other laboratories have defined the role of fungal HKs as critical to growth and virulence (summarized in the work of Li et al. [63]). First identified in *C. albicans*, HKs have since been validated in important cell functions in *C. glabrata*, *C. lusitaniae*, *C. neoformans*, *Penicillium marneffei*, *A. fumigatus*, and *Blastomyces dermatitidis* and at least found in the genomes of *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Coccidioides immitis*. Functional roles of HKs in these organisms include dimorphism, growth, adaptation to stress, cell wall biosynthesis, biofilm formation, expression of adhesin proteins, triazole tolerance, and, at least in four of these organisms, virulence. The other issue raised in this chapter is to discern the entire population of candidiasis patients and to consider those therapies that may be efficacious in particular groups of patients. This information is critical to understand the risk factors that play a role in disease progression and to help stratify patients based on disease severity and susceptibility. This will also pave the way for effectively selecting drug targets and candidates that will best serve the needs of the patient populations that suffer from these fungal infections.

REFERENCES

1. **Ameen, M., and R. Arenas.** 2009. Developments in the management of mycetomas. *Clin. Exp. Dermatol.* **34:**1–7.
2. **Arikan, S., and J. H. Rex.** 2001. Nystatin LF (Aronex/Abbott). *Curr. Opin. Investig. Drugs* **2:**488–495.
3. **Arita, A., X. Zhou, T. P. Ellen, X. Liu, J. Bai, J. P. Rooney, A. Kurtz, C. B. Klein, W. Dai, T. J. Begley, and M. Costa.** 2009. A genome-wide deletion mutant screen identifies pathways affected by nickel sulfate in *Saccharomyces cerevisiae*. *BMC Genomics* **10:**524.
4. **Baetz, K., L. McHardy, K. Gable, T. Tarling, D. Reberioux, J. Bryan, R. J. Andersen, T. Dunn, P. Hieter, and M. Roberge.** 2004. Yeast genome-wide drug-induced haploinsufficiency screen to determine drug mode of action. *Proc. Natl. Acad. Sci. USA* **101:**4525–4530.
5. **Batova, M., V. Klobucnikova, Z. Oblasova, J. Gregan, P. Zahradnik, I. Hapala, J. Subik, and C. Schuller.** 2010. Chemogenomic and transcriptome analysis identifies mode of action of the chemosensitizing agent CTBT (7-chlorotetrazolo[5,1-c]benzo[1,2,4]triazine). *BMC Genomics* **11:**153.
6. **Bennett, J. E.** 2009. The changing face of febrile neutropenia—from monotherapy to moulds to mucositis. Management of mycoses in neutropenic patients: a brief history, 1960–2008. *J. Antimicrob. Chemother.* **63**(Suppl. 1)**:**i23–i26.
7. **Blignaut, E.** 2007. Oral candidiasis and oral yeast carriage among institutionalised South African paediatric HIV/AIDS patients. *Mycopathologia* **163:**67–73.
8. **Boechk, M., and K. Marr.** 2002. Infection in hematopoietic stem cell transplantation, p. 527–571. *In* R. H. Rubin and L. S. Young (ed.), *Clinical Approach to Infection in the Compromised Host*. Kluwer Academic/Plenum, New York, NY.
9. **Bonifaz, A., D. Vazquez-Gonzalez, and A. M. Perusquia-Ortiz.** 2010. Subcutaneous mycoses: chromoblastomycosis, sporotrichosis and mycetoma. *J. Dtsch. Dermatol. Ges.* **8:**619–627; quiz, 628.
10. **Boucher, H. W., G. H. Talbot, J. S. Bradley, J. E. Edwards, D. Gilbert, L. B. Rice, M. Scheld, B. Spellberg, and J. Bartlett.** 2009. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**1–12.
11. **Bougnoux, M. E., G. Kac, P. Aegerter, C. d'Enfert, J. Y. Fagon, and CandiRea Study Group.** 2008. Candidemia and candiduria in critically ill patients admitted to intensive care units in France: incidence, molecular diversity, management and outcome. *Intensive Care Med.* **34:**292–299.
12. **Bredel, M., and E. Jacoby.** 2004. Chemogenomics: an emerging strategy for rapid target and drug discovery. *Nat. Rev. Genet.* **5:**262–275.
13. **Bromuro, C., M. Romano, P. Chiani, F. Berti, M. Tontini, D. Proietti, E. Mori, A. Torosantucci, P. Costantino, R. Rappuoli, and A. Cassone.** 2010. Beta-glucan-CRM197 conjugates as candidates antifungal vaccines. *Vaccine* **28:**2615–2623.
14. **Cabib, E.** 1991. Differential inhibition of chitin synthetases 1 and 2 from *Saccharomyces cerevisiae* by polyoxin D and nikkomycins. *Antimicrob. Agents Chemother.* **35:**170–173.
15. **Cagnoni, P. J., T. J. Walsh, M. M. Prendergast, D. Bodensteiner, S. Hiemenz, R. N. Greenberg, C. A. Arndt, M. Schuster, N. Seibel, V. Yeldandi, and K. B. Tong.** 2000. Pharmacoeconomic analysis of liposomal amphotericin B versus conventional amphotericin B in the empirical treatment of persistently febrile neutropenic patients. *J. Clin. Oncol.* **18:**2476–2483.
16. **Canton, E., J. Peman, A. Valentin, A. Espinel-Ingroff, and M. Gobernado.** 2009. In vitro activities of echinocandins against *Candida krusei* determined by three methods: MIC and minimal fungicidal concentration measurements and time-kill studies. *Antimicrob. Agents Chemother.* **53:**3108–3111.
17. **Caston-Osorio, J. J., A. Rivero, and J. Torre-Cisneros.** 2008. Epidemiology of invasive fungal infection. *Int. J. Antimicrob. Agents* **32**(Suppl. 2)**:**S103–S109.
18. **Cegelski, L., G. R. Marshall, G. R. Eldridge, and S. J. Hultgren.** 2008. The biology and future prospects of antivirulence therapies. *Nat. Rev. Microbiol.* **6:**17–27.
19. **Chakrabarti, A., S. S. Chatterjee, and M. R. Shivaprakash.** 2008. Overview of opportunistic fungal infections in India. *Nippon Ishinkin Gakkai Zasshi* **49:**165–172.
20. **Chakraborty, N., A. Mukherjee, S. Santra, R. N. Sarkar, D. Banerjee, S. K. Guha, S. Chakraborty, and S. K. Bhattacharyya.** 2008. Current trends of opportunistic infections among HIV-seropositive patients from Eastern India. *Jpn. J. Infect. Dis.* **61:**49–53.
21. **Delmas, G., S. Park, Z. W. Chen, F. Tan, R. Kashiwazaki, L. Zarif, and D. S. Perlin.** 2002. Efficacy of orally delivered cochleates containing amphotericin B in a murine model of aspergillosis. *Antimicrob. Agents Chemother.* **46:**2704–2707.
22. **De Pauw, B., T. J. Walsh, J. P. Donnelly, D. A. Stevens, J. E. Edwards, T. Calandra, P. G. Pappas, J. Maertens, O. Lortholary, C. A. Kauffman, D. W. Denning, T. F. Patterson, G. Maschmeyer, J. Bille, W. E. Dismukes, R. Herbrecht, W. W. Hope, C. C. Kibbler, B. J. Kullberg, K. A. Marr, P. Munoz, F. C. Odds, J. R. Perfect, A. Restrepo, M. Ruhnke, B. H. Segal, J. D. Sobel, T. C. Sorrell, C. Viscoli, J. R. Wingard, T. Zaoutis, J. E. Bennett, European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group, and National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group.** 2008. Revised definitions of invasive fungal disease from the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group (EORTC/MSG) Consensus Group. *Clin. Infect. Dis.* **46:**1813–1821.
23. **Dominguez, J. M., and J. J. Martin.** 1998. Identification of elongation factor 2 as the essential protein targeted by

sordarins in *Candida albicans*. *Antimicrob. Agents Chemother.* **42:**2279–2283.
24. **Drewry, D. H., and S. S. Young.** 1999. Approaches to the design of combinatorial libraries. *Chemom. Intell. Lab. Syst.* **48:**1–20.
25. **Espinel-Ingroff, A.** 2009. Novel antifungal agents, targets or therapeutic strategies for the treatment of invasive fungal diseases: a review of the literature (2005–2009). *Rev. Iberoam. Micol.* **26:**15–22.
26. **Fidel, P. L., Jr.** 2006. *Candida*-host interactions in HIV disease: relationships in oropharyngeal candidiasis. *Adv. Dent. Res.* **19:**80–84.
27. **Fricker-Hidalgo, H., B. Lebeau, H. Pelloux, and R. Grillot.** 2004. Use of the BACTEC 9240 System with Mycosis-IC/F blood culture bottles for detection of fungemia. *J. Clin. Microbiol.* **42:**1855–1856. (Author's reply, **42:**1855–1856.)
28. **Gagne, J. J., and N. I. Goldfarb.** 2007. Candidemia in the in-patient setting: treatment options and economics. *Expert Opin. Pharmacother.* **8:**1643–1650.
29. **Garcia-Effron, G., S. K. Katiyar, S. Park, T. D. Edlind, and D. S. Perlin.** 2008. A naturally occurring proline-to-alanine amino acid change in Fks1p in *Candida parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis* accounts for reduced echinocandin susceptibility. *Antimicrob. Agents Chemother.* **52:**2305–2312.
30. **Gavalda, J., O. Len, R. San Juan, J. M. Aguado, J. Fortun, C. Lumbreras, A. Moreno, P. Munoz, M. Blanes, A. Ramos, G. Rufi, M. Gurgui, J. Torre-Cisneros, M. Montejo, M. Cuenca-Estrella, J. L. Rodriguez-Tudela, A. Pahissa, and RESITRA (Spanish Network for Research on Infection in Transplantation).** 2005. Risk factors for invasive aspergillosis in solid-organ transplant recipients: a case-control study. *Clin. Infect. Dis.* **41:**52–59.
31. **Ghannoum, M. A., H. G. Kim, and L. Long.** 2007. Efficacy of aminocandin in the treatment of immunocompetent mice with haematogenously disseminated fluconazole-resistant candidiasis. *J. Antimicrob. Chemother.* **59:**556–559.
32. **Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. Andre, A. P. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K. D. Entian, P. Flaherty, F. Foury, D. J. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J. H. Hegemann, S. Hempel, Z. Herman, D. F. Jaramillo, D. E. Kelly, S. L. Kelly, P. Kotter, D. LaBonte, D. C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S. L. Ooi, J. L. Revuelta, C. J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D. D. Shoemaker, S. Sookhai-Mahadeo, R. K. Storms, J. N. Strathern, G. Valle, M. Voet, G. Volckaert, C. Y. Wang, T. R. Ward, J. Wilhelmy, E. A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J. D. Boeke, M. Snyder, P. Philippsen, R. W. Davis, and M. Johnston.** 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418:**387–391.
33. **Giaever, G., P. Flaherty, J. Kumm, M. Proctor, C. Nislow, D. F. Jaramillo, A. M. Chu, M. I. Jordan, A. P. Arkin, and R. W. Davis.** 2004. Chemogenomic profiling: identifying the functional interactions of small molecules in yeast. *Proc. Natl. Acad. Sci. USA* **101:**793–798.
34. **Groll, A. H., P. M. Shah, C. Mentzel, M. Schneider, G. Just-Nuebling, and K. Huebner.** 1996. Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. *J. Infect.* **33:**23–32.
35. **Guillon, R., F. Giraud, C. Loge, M. Le Borgne, C. Picot, F. Pagniez, and P. Le Pape.** 2009. Design of new antifungal agents: synthesis and evaluation of 1-[(1H-indol-5-ylmethyl)amino]-2-phenyl-3-(1H-1,2,4-triazol-1-yl) propan-2- ols. *Bioorg. Med. Chem. Lett.* **19:**5833–5836.
36. **Guillon, R., C. Loge, F. Pagniez, V. Ferchaud-Roucher, M. Duflos, C. Picot, and P. L. Pape.** 2010. Synthesis and in vitro antifungal evaluation of 2-(2,4-difluorophenyl)-1--[(1H-indol-3-ylmethyl)methylamino]-3-(1H-1,2,4-triazol-1-yl)propan-2-ols. *J. Enzyme Inhib. Med. Chem.* **26:**261–269.
37. **Hachem, R., H. Hanna, D. Kontoyiannis, Y. Jiang, and I. Raad.** 2008. The changing epidemiology of invasive candidiasis: *Candida glabrata* and *Candida krusei* as the leading causes of candidemia in hematologic malignancy. *Cancer* **112:**2493–2499.
38. **Hamza, O. J., M. I. Matee, M. J. Moshi, E. N. Simon, F. Mugusi, F. H. Mikx, W. H. Helderman, A. J. Rijs, A. J. van der Ven, and P. E. Verweij.** 2008. Species distribution and in vitro antifungal susceptibility of oral yeast isolates from Tanzanian HIV-infected patients with primary and recurrent oropharyngeal candidiasis. *BMC Microbiol.* **8:**135.
39. **Hanadate, T., M. Tomishima, N. Shiraishi, D. Tanabe, H. Morikawa, D. Barrett, S. Matsumoto, K. Ohtomo, and K. Maki.** 2009. FR290581, a novel sordarin derivative: synthesis and antifungal activity. *Bioorg. Med. Chem. Lett.* **19:**1465–1468.
40. **Harbarth, S., C. Ruef, P. Francioli, A. Widmer, and D. Pittet.** 1999. Nosocomial infections in Swiss university hospitals: a multi-centre survey and review of the published experience. Swiss-Noso Network. *Schweiz. Med. Wochenschr.* **129:**1521–1528.
41. **Hasenoehrl, A., T. Galic, G. Ergovic, N. Marsic, M. Skerlev, J. Mittendorf, U. Geschke, A. Schmidt, and W. Schoenfeld.** 2006. In vitro activity and in vivo efficacy of icofungipen (PLD-118), a novel oral antifungal agent, against the pathogenic yeast *Candida albicans*. *Antimicrob. Agents Chemother.* **50:**3011–3018.
42. **Hector, R. F., B. L. Zimmer, and D. Pappagianis.** 1990. Evaluation of nikkomycins X and Z in murine models of coccidioidomycosis, histoplasmosis, and blastomycosis. *Antimicrob. Agents Chemother.* **34:**587–593.
43. **Hiemenz, J. W., and T. J. Walsh.** 1996. Lipid formulations of amphotericin B: recent progress and future directions. *Clin. Infect. Dis.* **22**(Suppl. 2)**:**S133–S144.
44. **Hillenmeyer, M. E., E. Fung, J. Wildenhain, S. E. Pierce, S. Hoon, W. Lee, M. Proctor, R. P. St. Onge, M. Tyers, D. Koller, R. B. Altman, R. W. Davis, C. Nislow, and G. Giaever.** 2008. The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* **320:**362–365.
45. **Hodgetts, S., L. Nooney, R. Al-Akeel, A. Curry, S. Awad, R. Matthews, and J. Burnie.** 2008. Efungumab and caspofungin: pre-clinical data supporting synergy. *J. Antimicrob. Chemother.* **61:**1132–1139.
46. **Horn, D. L., D. Neofytos, E. J. Anaissie, J. A. Fishman, W. J. Steinbach, A. J. Olyaei, K. A. Marr, M. A. Pfaller, C. H. Chang, and K. M. Webster.** 2009. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. *Clin. Infect. Dis.* **48:**1695–1703.
47. **Ibrahim, A. S., B. J. Spellberg, V. Avanesian, Y. Fu, and J. E. Edwards, Jr.** 2006. The anti-*Candida* vaccine based on the recombinant N-terminal domain of Als1p is broadly active against disseminated candidiasis. *Infect. Immun.* **74:**3039–3041.
47a. **Infectious Diseases Society of America.** 2010. The 10 × '20 initiative: pursuing a global commitment to develop 10 new antibacterial drugs by 2020. *Clin. Infect. Dis.* **50:**1081–1083.

48. **Jabra-Rizk, M. A., W. A. Falkler, Jr., C. O. Enwonwu, D. I. Onwujekwe, Jr., W. G. Merz, and T. F. Meiller.** 2001. Prevalence of yeast among children in Nigeria and the United States. *Oral Microbiol. Immunol.* **16:**383–385.
49. **Jiang, B., D. Xu, J. Allocco, C. Parish, J. Davison, K. Veillette, S. Sillaots, W. Hu, R. Rodriguez-Suarez, S. Trosok, L. Zhang, Y. Li, F. Rahkhoodaee, T. Ransom, N. Martel, H. Wang, D. Gauvin, J. Wiltsie, D. Wisniewski, S. Salowe, J. N. Kahn, M. J. Hsu, R. Giacobbe, G. Abruzzo, A. Flattery, C. Gill, P. Youngman, K. Wilson, G. Bills, G. Platas, F. Pelaez, M. T. Diez, S. Kauffman, J. Becker, G. Harris, P. Liberator, and T. Roemer.** 2008. PAP inhibitor with in vivo efficacy identified by *Candida albicans* genetic profiling of natural products. *Chem. Biol.* **15:**363–374.
50. **Jung, S. H., D. H. Lim, S. H. Jung, J. E. Lee, K. S. Jeong, H. Seong, and B. C. Shin.** 2009. Amphotericin B-entrapping lipid nanoparticles and their in vitro and in vivo characteristics. *Eur. J. Pharm. Sci.* **37:**313–320.
51. **Justice, M. C., M. J. Hsu, B. Tse, T. Ku, J. Balkovec, D. Schmatz, and J. Nielsen.** 1998. Elongation factor 2 as a novel target for selective inhibition of fungal protein synthesis. *J. Biol. Chem.* **273:**3148–3151.
52. **Kakeya, H., Y. Miyazaki, H. Senda, T. Kobayashi, M. Seki, K. Izumikawa, K. Yanagihara, Y. Yamamoto, T. Tashiro, and S. Kohno.** 2008. Efficacy of SPK-843, a novel polyene antifungal, in comparison with amphotericin B, liposomal amphotericin B, and micafungin against murine pulmonary aspergillosis. *Antimicrob. Agents Chemother.* **52:** 1868–1870.
53. **Kamai, Y., M. Kakuta, T. Shibayama, T. Fukuoka, and S. Kuwahara.** 2005. Antifungal activities of R-135853, a sordarin derivative, in experimental candidiasis in mice. *Antimicrob. Agents Chemother.* **49:**52–56.
54. **Kang, T. H., E. I. Hwang, B. S. Yun, K. D. Park, B. M. Kwon, C. S. Shin, and S. U. Kim.** 2007. Inhibition of chitin synthases and antifungal activities by 2′-benzoyloxycinnamaldehyde from *Pleuropterus ciliinervis* and its derivatives. *Biol. Pharm. Bull.* **30:**598–602.
55. **Kang, T. H., E. I. Hwang, B. S. Yun, C. S. Shin, and S. U. Kim.** 2008. Chitin synthase 2 inhibitory activity of *O*-methyl pisiferic acid and 8,20-dihydroxy-9(11),13-abietadien-12-one, isolated from *Chamaecyparis pisifera*. *Biol. Pharm. Bull.* **31:**755–759.
56. **Kitamura, A., K. Someya, M. Hata, R. Nakajima, and M. Takemura.** 2009. Discovery of a small-molecule inhibitor of β-1,6-glucan synthesis. *Antimicrob. Agents Chemother.* **53:**670–677.
57. **Knaus, W. A., D. P. Wagner, E. A. Draper, J. E. Zimmerman, M. Bergner, P. G. Bastos, C. A. Sirio, D. J. Murphy, T. Lotring, and A. Damiano.** 1991. The APACHE III prognostic system. Risk prediction of hospital mortality for critically ill hospitalized adults. *Chest* **100:**1619–1636.
58. **Kontoyiannis, D. P., K. A. Marr, B. J. Park, B. D. Alexander, E. J. Anaissie, T. J. Walsh, J. Ito, D. R. Andes, J. W. Baddley, J. M. Brown, L. M. Brumble, A. G. Freifeld, S. Hadley, L. A. Herwaldt, C. A. Kauffman, K. Knapp, G. M. Lyon, V. A. Morrison, G. Papanicolaou, T. F. Patterson, T. M. Perl, M. G. Schuster, R. Walker, K. A. Wannemuehler, J. R. Wingard, T. M. Chiller, and P. G. Pappas.** 2010. Prospective surveillance for invasive fungal infections in hematopoietic stem cell transplant recipients, 2001–2006: overview of the Transplant-Associated Infection Surveillance Network (TRANSNET) Database. *Clin. Infect. Dis.* **50:**1091–1100.
59. **Krcmery, V., and A. J. Barnes.** 2002. Non-albicans *Candida* spp. causing fungaemia: pathogenicity and antifungal resistance. *J. Hosp. Infect.* **50:**243–260.
60. **Lass-Florl, C.** 2009. The changing face of epidemiology of invasive fungal disease in Europe. *Mycoses* **52:**197–205.
61. **Lehrnbecher, T., C. Frank, K. Engels, S. Kriener, A. H. Groll, and D. Schwabe.** 2010. Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. *J. Infect.* **61:**259–265.
62. **Lewis, R. E.** 2007. Pharmacodynamic implications for use of antifungal agents. *Curr. Opin. Pharmacol.* **7:**491–497.
63. **Li, D., O. A. Agrellos, and R. Calderone.** 2010. Histidine kinases keep fungi safe and vigorous. *Curr. Opin. Microbiol.* **13:**424–430.
64. **Li, J. W., and J. C. Vederas.** 2009. Drug discovery and natural products: end of an era or an endless frontier? *Science* **325:**161–165.
65. **Liu, T. T., R. E. Lee, K. S. Barker, R. E. Lee, L. Wei, R. Homayouni, and P. D. Rogers.** 2005. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob. Agents Chemother.* **49:**2226–2236.
66. **Lortholary, O., D. W. Denning, and B. Dupont.** 1999. Endemic mycoses: a treatment update. *J. Antimicrob. Chemother.* **43:**321–331.
67. **Lortholary, O., M. Desnos-Ollivier, K. Sitbon, A. Fontanet, S. Bretagne, F. Dromer, and the French Mycosis Study Group.** 2011. Recent exposure to caspofungin or fluconazole influences the epidemiology of candidemia: a prospective multicenter study involving 2,441 patients. *Antimicrob. Agents Chemother.* **55:**532–538.
68. **Micol, R., A. Tajahmady, O. Lortholary, S. Balkan, C. Quillet, J. P. Dousset, H. Chanroeun, Y. Madec, A. Fontanet, and Y. Yazdanpanah.** 2010. Cost-effectiveness of primary prophylaxis of AIDS associated cryptococcosis in Cambodia. *PLoS One* **5:**e13856.
69. **Miller, L. G., R. A. Hajjeh, and J. E. Edwards, Jr.** 2001. Estimating the cost of nosocomial candidemia in the United States. *Clin. Infect. Dis.* **32:**1110.
70. **Mitsuyama, J., N. Nomura, K. Hashimoto, E. Yamada, H. Nishikawa, M. Kaeriyama, A. Kimura, Y. Todo, and H. Narita.** 2008. In vitro and in vivo antifungal activities of T-2307, a novel arylamidine. *Antimicrob. Agents Chemother.* **52:**1318–1324.
71. **Mosmann, T.** 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65:**55–63.
72. **Nadagir, S. D., S. K. Chunchanur, L. H. Halesh, K. Yasmeen, M. R. Chandrasekhar, and B. S. Patil.** 2008. Significance of isolation and drug susceptibility testing of non-*Candida albicans* species causing oropharyngeal candidiasis in HIV patients. *Southeast Asian J. Trop. Med. Public Health* **39:**492–495.
73. **Nakamata, K., T. Kurita, M. S. Bhuiyan, K. Sato, Y. Noda, and K. Yoda.** 2007. KEG1/YFR042w encodes a novel Kre6-binding endoplasmic reticulum membrane protein responsible for beta-1,6-glucan synthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **282:**34315–34324.
74. **Neofytos, D., D. Horn, E. Anaissie, W. Steinbach, A. Olyaei, J. Fishman, M. Pfaller, C. Chang, K. Webster, and K. Marr.** 2009. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of Multicenter Prospective Antifungal Therapy (PATH) Alliance registry. *Clin. Infect. Dis.* **48:**265–273.
75. **Niimi, M., N. A. Firth, and R. D. Cannon.** 2010. Antifungal drug resistance of oral fungi. *Odontology* **98:**15–25.
76. **Nishikaku, A., A. Melo, and A. Colombo.** 2010. Geographic trends in invasive candidiasis. *Curr. Fungal Infect.* **4:**210–218.
77. **Nomura, K., K. Kawasugi, and T. Morimoto.** 2006. Cost-effectiveness analysis of antifungal treatment for patients on chemotherapy. *Eur. J. Cancer Care (Engl.)* **15:** 44–50.

78. **Odds, F. C., A. J. Brown, and N. A. Gow.** 2003. Antifungal agents: mechanisms of action. *Trends Microbiol.* **11:** 272–279.
79. **Oh, J., E. Fung, U. Schlecht, R. W. Davis, G. Giaever, R. P. St. Onge, A. Deutschbauer, and C. Nislow.** 2010. Gene annotation and drug target discovery in *Candida albicans* with a tagged transposon mutant collection. *PLoS Pathog.* **6:**e1001140.
80. **Okome-Nkoumou, M., M. E. Mbounja-Loclo, and M. Kombila.** 2000. Spectrum of opportunistic infections in subjects infected with HIV at Libreville, Gabon. *Sante* **10:**329–337. (In French.)
81. **Olaechea, P. M., M. Palomar, C. Leon-Gil, F. Alvarez-Lerma, R. Jorda, J. Nolla-Salas, M. A. Leon-Regidor, and EPCAN Study Group.** 2004. Economic impact of *Candida* colonization and *Candida* infection in the critically ill patient. *Eur. J. Clin. Microbiol. Infect. Dis.* **23:** 323–330.
82. **Ostrosky-Zeichner, L., A. Casadevall, J. N. Galgiani, F. C. Odds, and J. H. Rex.** 2010. An insight into the antifungal pipeline: selected new molecules and beyond. *Nat. Rev. Drug Discov.* **9:**719–727.
83. **Pachl, J., P. Svoboda, F. Jacobs, K. Vandewoude, B. van der Hoven, P. Spronk, G. Masterson, M. Malbrain, M. Aoun, J. Garbino, J. Takala, L. Drgona, J. Burnie, R. Matthews, and Mycograb Invasive Candidiasis Study Group.** 2006. A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. *Clin. Infect. Dis.* **42:**1404–1413.
84. **Pappas, P. G., B. D. Alexander, D. R. Andes, S. Hadley, C. A. Kauffman, A. Freifeld, E. J. Anaissie, L. M. Brumble, L. Herwaldt, J. Ito, D. P. Kontoyiannis, G. M. Lyon, K. A. Marr, V. A. Morrison, B. J. Park, T. F. Patterson, T. M. Perl, R. A. Oster, M. G. Schuster, R. Walker, T. J. Walsh, K. A. Wannemuehler, and T. M. Chiller.** 2010. Invasive fungal infections among organ transplant recipients: results of the Transplant-Associated Infection Surveillance Network (TRANSNET). *Clin. Infect. Dis.* **50:** 1101–1111.
85. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, J. D. Sobel, and Infectious Diseases Society of America.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.
86. **Parish, C. A., S. K. Smith, K. Calati, D. Zink, K. Wilson, T. Roemer, B. Jiang, D. Xu, G. Bills, G. Platas, F. Pelaez, M. T. Diez, N. Tsou, A. E. McKeown, R. G. Ball, M. A. Powles, L. Yeung, P. Liberator, and G. Harris.** 2008. Isolation and structure elucidation of parnafungins, antifungal natural products that inhibit mRNA polyadenylation. *J. Am. Chem. Soc.* **130:**7060–7066.
87. **Peman, J., and R. Zaragoza.** 2010. Current diagnostic approaches to invasive candidiasis in critical care settings. *Mycoses* **53:**424–433.
88. **Pfaller, M. A., L. Boyken, R. J. Hollis, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2006. In vitro susceptibilities of *Candida* spp. to caspofungin: four years of global surveillance. *J. Clin. Microbiol.* **44:**760–763.
89. **Pfaller, M. A., M. Castanheira, S. A. Messer, G. J. Moet, and R. N. Jones.** 2010. Variation in *Candida* spp. distribution and antifungal resistance rates among bloodstream infection isolates by patient age: report from the SENTRY Antimicrobial Surveillance Program (2008–2009). *Diagn. Microbiol. Infect. Dis.* **68:**278–283.
90. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
91. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, H. Bijie, D. Dzierzanowska, N. N. Klimko, V. Letscher-Bru, M. Lisalova, K. Muehlethaler, C. Rennison, M. Zaidi, and Global Antifungal Surveillance Group.** 2009. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: 10.5-year analysis of susceptibilities of noncandidal yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J. Clin. Microbiol.* **47:** 117–123.
92. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, D. Ellis, V. Tullio, A. Rodloff, W. Fu, T. A. Ling, and the Global Antifungal Surveillance Group.** 2010. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *J. Clin. Microbiol.* **48:**1366–1377.
93. **Pfaller, M. A., D. J. Diekema, and International Fungal Surveillance Participant Group.** 2004. Twelve years of fluconazole in clinical practice: global trends in species distribution and fluconazole susceptibility of bloodstream isolates of *Candida*. *Clin. Microbiol. Infect.* **10**(Suppl. 1)**:** 11–23.
94. **Pfaller, M. A., S. A. Messer, N. Georgopapadakou, L. A. Martell, J. M. Besterman, and D. J. Diekema.** 2009. Activity of MGCD290, a Hos2 histone deacetylase inhibitor, in combination with azole antifungals against opportunistic fungal pathogens. *J. Clin. Microbiol.* **47:** 3797–3804.
95. **Pienaar, E. D., T. Young, and H. Holmes.** 2010. Interventions for the prevention and management of oropharyngeal candidiasis associated with HIV infection in adults and children. *Cochrane Database Syst. Rev.* **11:** CD003940.
96. **Playford, E. G., J. Lipman, and T. C. Sorrell.** 2010. Prophylaxis, empirical and preemptive treatment of invasive candidiasis. *Curr. Opin. Crit. Care* **16:**470–474.
97. **Prentice, H. G., I. M. Hann, R. Herbrecht, M. Aoun, S. Kvaloy, D. Catovsky, C. R. Pinkerton, S. A. Schey, F. Jacobs, A. Oakhill, R. F. Stevens, P. J. Darbyshire, and B. E. Gibson.** 1997. A randomized comparison of liposomal versus conventional amphotericin B for the treatment of pyrexia of unknown origin in neutropenic patients. *Br. J. Haematol.* **98:**711–718.
98. **Qin, Z., J. Zhang, B. Xu, L. Chen, Y. Wu, X. Yang, X. Shen, S. Molin, A. Danchin, H. Jiang, and D. Qu.** 2006. Structure-based discovery of inhibitors of the YycG histidine kinase: new chemical leads to combat *Staphylococcus epidermidis* infections. *BMC Microbiol.* **6:**96.
99. **Rentz, A. M., M. T. Halpern, and R. Bowden.** 1998. The impact of candidemia on length of hospital stay, outcome, and overall cost of illness. *Clin. Infect. Dis.* **27:** 781–788.
100. **Repetto, G., A. del Peso, and J. L. Zurita.** 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nat. Protoc.* **3:**1125–1131.
101. **Rijnders, B. J., J. J. Cornelissen, L. Slobbe, M. J. Becker, J. K. Doorduijn, W. C. Hop, E. J. Ruijgrok, B. Lowenberg, A. Vulto, P. J. Lugtenburg, and S. de Marie.** 2008. Aerosolized liposomal amphotericin B for the prevention of invasive pulmonary aspergillosis during prolonged neutropenia: a randomized, placebo-controlled trial. *Clin. Infect. Dis.* **46:**1401–1408.
102. **Rodriguez-Suarez, R., D. Xu, K. Veillette, J. Davison, S. Sillaots, S. Kauffman, W. Hu, J. Bowman, N. Martel,**

S. Trosok, H. Wang, L. Zhang, L. Y. Huang, Y. Li, F. Rahkhoodaee, T. Ransom, D. Gauvin, C. Douglas, P. Youngman, J. Becker, B. Jiang, and T. Roemer. 2007. Mechanism-of-action determination of GMP synthase inhibitors and target validation in *Candida albicans* and *Aspergillus fumigatus*. *Chem. Biol.* **14:**1163–1175.

103. **Roemer, T., B. Jiang, J. Davison, T. Ketela, K. Veillette, A. Breton, F. Tandia, A. Linteau, S. Sillaots, C. Marta, N. Martel, S. Veronneau, S. Lemieux, S. Kauffman, J. Becker, R. Storms, C. Boone, and H. Bussey.** 2003. Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. *Mol. Microbiol.* **50:**167–181.
104. **Sampaio Camargo, T. Z., A. R. Marra, C. V. Silva, M. F. Cardoso, M. D. Martino, L. F. Camargo, and L. Correa.** 2010. Secular trends of candidemia in a tertiary care hospital. *Am. J. Infect. Control* **38:**546–551.
105. **Santangelo, R., P. Paderu, G. Delmas, Z. W. Chen, R. Mannino, L. Zarif, and D. S. Perlin.** 2000. Efficacy of oral cochleate-amphotericin B in a mouse model of systemic candidiasis. *Antimicrob. Agents Chemother.* **44:** 2356–2360.
106. **Seifert, H., U. Aurbach, D. Stefanik, and O. Cornely.** 2007. In vitro activities of isavuconazole and other antifungal agents against *Candida* bloodstream isolates. *Antimicrob. Agents Chemother.* **51:**1818–1821.
107. **Smith, A. M., R. Ammar, C. Nislow, and G. Giaever.** 2010. A survey of yeast genomic assays for drug and target discovery. *Pharmacol. Ther.* **127:**156–164.
108. **Sobel, J. D.** 2007. Vulvovaginal candidosis. *Lancet* **369:**1961–1971.
109. **Sopko, R., D. Huang, N. Preston, G. Chua, B. Papp, K. Kafadar, M. Snyder, S. G. Oliver, M. Cyert, T. R. Hughes, C. Boone, and B. Andrews.** 2006. Mapping pathways and phenotypes by systematic gene overexpression. *Mol. Cell* **21:**319–330.
110. **Sorbera, L. A., J. Bartroli, and J. Castaner.** 2003. Albaconazole. *Drugs Future* **28:**529–537.
111. **Spellberg, B. J., S. G. Filler, and J. E. Edwards, Jr.** 2006. Current treatment strategies for disseminated candidiasis. *Clin. Infect. Dis.* **42:**244–251.
112. **Spellberg, B. J., A. S. Ibrahim, V. Avanesian, Y. Fu, C. Myers, Q. T. Phan, S. G. Filler, M. R. Yeaman, and J. E. Edwards, Jr.** 2006. Efficacy of the anti-*Candida* rAls3p-N or rAls1p-N vaccines against disseminated and mucosal candidiasis. *J. Infect. Dis.* **194:**256–260.
113. **Tolman, J. A., N. P. Wiederhold, J. T. McConville, L. K. Najvar, R. Bocanegra, J. I. Peters, J. J. Coalson, J. R. Graybill, T. F. Patterson, and R. O. Williams III.** 2009. Inhaled voriconazole for prevention of invasive pulmonary aspergillosis. *Antimicrob. Agents Chemother.* **53:**2613–2615.
114. **Trofa, D., A. Gacser, and J. D. Nosanchuk.** 2008. *Candida parapsilosis*, an emerging fungal pathogen. *Clin. Microbiol. Rev.* **21:**606–625.
115. **Uppuluri, P., J. Nett, J. Heitman, and D. Andes.** 2008. Synergistic effect of calcineurin inhibitors and fluconazole against *Candida albicans* biofilms. *Antimicrob. Agents Chemother.* **52:**1127–1132.
116. **van der Meer, J. W., F. L. van de Veerdonk, L. A. Joosten, B. J. Kullberg, and M. G. Netea.** 2010. Severe *Candida* spp. infections: new insights into natural immunity. *Int. J. Antimicrob. Agents* **36**(Suppl. 2)**:**S58–S62.
117. **van Gool, R.** 2001. The cost of treating systemic fungal infections. *Drugs* **61**(Suppl. 1)**:**49–56.
118. **Vogel, H. G.** 2007. Assays for insulin and insulin-like metabolic activity, p. 1415–1416. *In* H. G. Vogel (ed.), *Drug Discovery and Evaluation: Pharmacological Assays*, 3rd ed. Springer, New York, NY.
119. **Walsh, T. J., J. Hiemenz, and P. A. Pizzo.** 1994. Evolving risk factors for invasive fungal infections—all neutropenic patients are not the same. *Clin. Infect. Dis.* **18:** 793–798.
120. **Warn, P. A., A. Sharp, G. Morrissey, and D. W. Denning.** 2005. Activity of aminocandin (IP960) compared with amphotericin B and fluconazole in a neutropenic murine model of disseminated infection caused by a fluconazole-resistant strain of *Candida tropicalis*. *J. Antimicrob. Chemother.* **56:**590–593.
121. **Warn, P. A., A. Sharp, G. Morrissey, and D. W. Denning.** 2010. Activity of aminocandin (IP960; HMR3270) compared with amphotericin B, itraconazole, caspofungin and micafungin in neutropenic murine models of disseminated infection caused by itraconazole-susceptible and -resistant strains of *Aspergillus fumigatus*. *Int. J. Antimicrob. Agents* **35:**146–151.
122. **Weig, M., and A. J. Brown.** 2007. Genomics and the development of new diagnostics and anti-*Candida* drugs. *Trends Microbiol.* **15:**310–317.
123. **Wertheimer, A. I., T. M. Santella, and H. J. Lauver.** 2004. Successful public/private donation programs: a review of the Diflucan Partnership Program in South Africa. *J. Int. Assoc. Physicians AIDS Care* **3:**74–79, 84–85.
124. **Widmer, F., L. C. Wright, D. Obando, R. Handke, R. Ganendren, D. H. Ellis, and T. C. Sorrell.** 2006. Hexadecylphosphocholine (miltefosine) has broad-spectrum fungicidal activity and is efficacious in a mouse model of cryptococcosis. *Antimicrob. Agents Chemother.* **50:** 414–421.
125. **Wilson, L. S., C. M. Reyes, M. Stolpman, J. Speckman, K. Allen, and J. Beney.** 2002. The direct cost and incidence of systemic fungal infections. *Value Health* **5:**26–34.
126. **Xu, D., B. Jiang, T. Ketela, S. Lemieux, K. Veillette, N. Martel, J. Davison, S. Sillaots, S. Trosok, C. Bachewich, H. Bussey, P. Youngman, and T. Roemer.** 2007. Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans*. *PLoS Pathog.* **3:**e92.
127. **Yamada, E., H. Nishikawa, N. Nomura, and J. Mitsuyama.** 2010. T-2307 shows efficacy in a murine model of *Candida glabrata* infection despite in vitro trailing growth phenomena. *Antimicrob. Agents Chemother.* **54:** 3630–3634.
128. **Yim, N. H., E. I. Hwang, B. S. Yun, K. D. Park, J. S. Moon, S. H. Lee, N. D. Sung, and S. U. Kim.** 2008. Sesquiterpene furan compound CJ-01, a novel chitin synthase 2 inhibitor from Chloranthus japonicus SIEB. *Biol. Pharm. Bull.* **31:**1041–1044.
129. **Zarif, L., J. R. Graybill, D. Perlin, L. Najvar, R. Bocanegra, and R. J. Mannino.** 2000. Antifungal activity of amphotericin B cochleates against *Candida albicans* infection in a mouse model. *Antimicrob. Agents Chemother.* **44:**1463–1469.
130. **Zhang, X., P. A. Reichart, and Y. Song.** 2009. Oral manifestations of HIV/AIDS in China: a review. *Oral Maxillofac. Surg.* **13:**63–68.
131. **Zilberberg, M. D., M. H. Kollef, H. Arnold, A. Labelle, S. T. Micek, S. Kothari, and A. F. Shorr.** 2010. Inappropriate empiric antifungal therapy for candidemia in the ICU and hospital resource utilization: a retrospective cohort study. *BMC Infect. Dis.* **10:**150.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch25

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Multidrug Resistance Transcriptional Regulatory Networks in *Candida*

P. DAVID ROGERS AND KATHERINE S. BARKER

Candida species are an important cause of both mucosal and invasive opportunistic infections among immunocompromised patient populations, such as cancer patients receiving cytotoxic chemotherapy, solid-organ and bone marrow transplant patients receiving immunosuppressant therapy, and patients infected with human immunodeficiency virus (HIV) and suffering from AIDS. *Candida albicans* remains the most common cause of fungal infections in humans; however, over the past two decades, there has been an increase in non-*C. albicans* species causing infection (8, 71). Several classes of antifungal agents are available for the treatment of mucosal and invasive fungal infections in these patient populations, including the polyenes, the pyrimidine analog flucytosine, the azoles, and, most recently, the echinocandins (71).

The polyene antifungals such as amphotericin B and nystatin bind to the fungal sterol ergosterol in the cell membrane, resulting in pore formation, leakage of intracellular contents, and, ultimately, cell death. These agents are fungicidal against most *Candida* species, yet their clinical utility is limited by route of administration and significant toxicity. Among polyenes, only amphotericin B is used for treatment of systemic infections, and it is only available for intravenous administration. Amphotericin B is associated with significant nephrotoxicity and infusion-related reactions (30, 55). Newer lipid-based formulations of this agent are associated with less toxicity but are substantially more expensive (101).

The azole antifungals bind to and inhibit the activity of lanosterol demethylase, a key enzyme in the ergosterol biosynthesis pathway. Azoles exhibit good activity against *Candida albicans* but are less active against *Candida glabrata* and *Candida krusei*. Some azoles, such as fluconazole, are available in both oral and intravenous formulations. While this antifungal class has only modest toxicity, the azoles are associated with a plethora of drug-drug interactions. Moreover, the azoles are not fungicidal, and clinical resistance is problematic in certain patient populations, especially among AIDS patients on prolonged courses of therapy for oropharyngeal candidiasis (OPC) (42, 89).

Flucytosine is taken up by a fungal specific transporter and converted to 5-fluorouracil (5-FU), which is incorporated into DNA and RNA and interferes with DNA and protein synthesis. This agent is associated with significant toxicity, such as bone marrow suppression (32). The echinocandins inhibit 1,3-beta-D-glucan synthase, thus interfering with cell wall synthesis. These antifungals are fungicidal, are associated with modest toxicity, and have few drug-drug interactions (103).

The development of clinical antifungal resistance has been reported for all four classes of antifungal agents and generally involves the selection of a subpopulation of fungal cells that harbor specific resistance-conferring mutations. Resistance to amphotericin B, although rare, is most often caused by mutations leading to altered membrane ergosterol content, thus reducing the interaction between the cell membrane and the drug (32, 47, 116). Flucytosine resistance is associated with a mutation in the gene encoding uracil phosphoribosyltransferase (*FUR1*) and is thought to result in reduced conversion of 5-FU to the toxic metabolite 5-fluorouridine monophosphate. Moreover, mutations in cytosine deaminase (*FCA1*) have been associated with resistance to flucytosine in clinical isolates (89, 91). Resistance to flucytosine occurs at a high frequency and precludes the use of this agent as monotherapy. Resistance to the echinocandins is due to mutations in the gene encoding 1,3-beta-D-glucan synthase interfering with the ability of the drug to inhibit cell wall synthesis (73, 103). Although uncommon to date, reports of clinical resistance to the echinocandins continue to emerge (10, 15, 20, 31, 45).

Azole resistance is the most common and clinically significant antifungal resistance and can be caused by a variety of mechanisms. Fluconazole and other azole antifungal agents have proven to be effective in the management of OPC; however, with increased use of these agents, treatment failures have occurred that have been associated with the emergence of azole-resistant strains of *C. albicans* (62, 116). The repetition and lengthy duration of therapy for OPC in this patient population have led to an increased

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incidence of treatment failures secondary to the emergence of azole resistance (81). While the use of highly active antiretroviral therapy (HAART) has reduced the frequency of OPC among AIDS patients in the United States (58), poor compliance, lack of access to HAART, and antiretroviral therapy failure have led to continued cases of OPC in this patient population (47, 107) and will likely contribute to an increase in this problem among AIDS patients worldwide. Moreover, recent reports have highlighted a paradoxical OPC infection rate of 30% in HIV-infected individuals who have shown improvements in $CD4^+$ counts and have been classified as "immune reconstituted" (29). Furthermore, the widespread use of fluconazole in the Third World is likely to maintain pressure on *C. albicans* to develop resistance. Recent surveillance data bear this out, as the highest incidences of fluconazole resistance in *C. albicans* are in North America and in Africa and the Middle East (74).

Multiple mechanisms have been shown to contribute to the development of azole resistance. Mutations in the gene encoding lanosterol demethylase (*ERG11*) can result in reduced azole binding and increased resistance (28, 72, 87). Mutations in the gene encoding sterol Δ5,6 desaturase (*ERG3*) prevent the conversion of 14α-methylated sterols into toxic 3,6-diol metabolites (43, 44, 67). Overexpression of the *ERG11* gene itself can result in resistance, presumably through increased production of the azole target, lanosterol demethylase (83, 115). Azole resistance can also be due to increased expression of genes encoding two classes of transporters (26, 54, 83, 115, 116). In *C. albicans*, the overexpression of the ATP-binding cassette (ABC) transporter genes *CDR1* and *CDR2* can confer cross-resistance to all azole antifungals, whereas overexpression of the major facilitator superfamily (MFS) transporter gene *MDR1* confers resistance specifically to the triazole fluconazole and its derivative voriconazole. Overexpression of the lanosterol demethylase gene and drug transporters has recently been shown to be due to mutations in specific transcription factors (17, 23, 63). This chapter focuses on the transcriptional regulation of azole antifungal resistance as well as transcriptional regulators that influence azole susceptibility in *Candida* species.

ALTERATIONS IN DRUG TRANSPORT AS A MECHANISM OF AZOLE RESISTANCE

There are two classes of drug transporters involved in azole antifungal resistance in yeasts, the MFS and ABC transporters (26, 54, 83, 115, 116). The MFS transporters utilize the proton gradient generated across the plasma membrane for drug efflux, whereas the ABC transporters hydrolyze ATP for drug efflux. Much of our understanding of azole antifungal resistance in *Candida* species has been greatly facilitated by the study of the pleiotropic drug resistance phenotype in *Saccharomyces cerevisiae*, which is largely driven by ABC transporters such as Pdr5p (64).

The first transporter implicated in antifungal resistance in *Candida* species was an efflux pump of the MFS from *Candida albicans*. The gene encoding this transporter, originally designated BENr, was discovered through its ability to confer resistance to the antifungal compound benomyl when expressed in *S. cerevisiae* (6, 25). Further studies showed that it could confer resistance to other compounds, including 4-nitroquinoline-*N*-oxide, benztriazoles, cycloheximide, methotrexate, and sulfomethuron methyl as well as the clinically used azole antifungal agent fluconazole and its derivative voriconazole. Based on the ability of this transporter to impart resistance to a wide range of structurally dissimilar compounds with unrelated mechanisms of action, the gene was renamed *MDR1* for multidrug resistance. Interestingly, Mdr1p does not influence resistance to the triazole itraconazole or either of the imidazoles ketoconazole and clotrimazole (66, 83).

In fluconazole-susceptible strains, *MDR1* appears to be expressed at very low levels (117). However, gene expression is induced upon exposure to certain compounds such as benomyl, diamide, and hydrogen peroxide. Importantly, the *MDR1* gene has been observed to be upregulated in many fluconazole-resistant clinical isolates obtained from AIDS patients receiving long-term therapy for OPC (26, 28, 54, 72, 83, 116). As expected, several of these isolates exhibit increased resistance to other Mdr1p substrates. Disruption of the *MDR1* gene in isolates such as these results in reduced resistance, demonstrating that this gene contributes to the azole resistance phenotype (117). Conversely, forced overexpression of *MDR1* results in increased resistance to fluconazole and other Mdr1p substrates, demonstrating that Mdr1 is itself sufficient to confer fluconazole resistance (36).

A second MFS transporter that influences fluconazole susceptibility was identified by complementation of a fluconazole-hypersusceptible *S. cerevisiae* mutant lacking the gene encoding the Pdr5p ABC transporter (9). Disruption of this gene, designated *FLU1* for fluconazole resistance, had a modest but measurable effect on azole susceptibility. To date, there have been no fluconazole-resistant clinical isolates found to overexpress this gene, suggesting that it may not be of clinical significance.

MFS transporters have been identified in other *Candida* species as well. *Candida dubliniensis*, a species closely related to *C. albicans*, possesses a homolog to *MDR1* (Cd*MDR1*) that represents an important mechanism of fluconazole resistance in this species (118). As in *C. albicans*, this gene has been found to be overexpressed in fluconazole-resistant clinical isolates, and disruption of Cd*MDR1* in such isolates reduces resistance. *MDR1* homologs have also been identified in *C. glabrata* and *C. tropicalis* (4, 13, 87). In *C. glabrata*, this gene has been designated Cg*FLR1*, and it is similar to *FLR1* in *S. cerevisiae* (13, 87). Cg*FLR1* can increase fluconazole resistance when expressed in *S. cerevisiae* but has not been associated with fluconazole resistance in clinical isolates. Likewise, the *C. tropicalis MDR1* homolog has been observed to be overexpressed in laboratory-derived fluconazole-resistant strains but not in clinical isolates (4).

The first ABC transporter associated with azole resistance in *C. albicans* was identified by its ability to complement cycloheximide resistance in an *S. cerevisiae* mutant lacking *PDR5* (75). This gene was designated *CDR1* for *Candida* drug resistance. A second, highly homologous ABC transporter gene (*CDR2*) was similarly identified in *C. albicans* (85). These transporters are capable of driving efflux of multiple substrates, including steroids, lipids, and a broad range of azole antifungal agents (98, 99). Other similar ABC transporters, such as *CDR3* and *CDR4*, have been identified in *C. albicans* but do not appear to influence azole susceptibility (3, 27).

Both *CDR1* and *CDR2* have been observed to be upregulated in many azole-resistant clinical isolates, and these transporters are often coregulated (54, 72, 83). Disruption of *CDR1* results in hypersusceptibility to azoles, other sterol biosynthesis inhibitors (terbinafine and amorolfine), and other inhibitors, such as cycloheximide and fluphenazine (84). Moreover, disruption of *CDR1* results in increased intracellular accumulation of fluconazole (84), and forced overexpression of either *CDR1* or *CDR2* results in increased

fluconazole resistance, demonstrating that these genes are major determinants of azole resistance in *C. albicans* (65). Deletion of *CDR1* in an azole-resistant clinical isolate resulted in a marked reduction in ketoconazole, fluconazole, and itraconazole resistance. Disruption of *CDR2* had only a modest effect in ketoconazole and fluconazole resistance and no effect on itraconazole resistance, demonstrating that *CDR1* plays the more significant role of these two transporters in azole resistance (109).

Homologs of *CDR1* and *CDR2* have been identified in other *Candida* species. *C. dubliniensis* has homologs of *CDR1* and *CDR2*, and both genes have been observed to be upregulated in azole-resistant clinical isolates. However, Cdr1p is nonfunctional in many strains due to a nonsense mutation in the *CDR1* gene. Overexpression of both genes in an *S. cerevisiae* mutant lacking Pdr5p increased fluconazole resistance, and disruption of *CDR1* in *C. dubliniensis* resulted in increase azole susceptibility (61).

Also of importance are the ABC transporters *CDR1*, *PDH1*, and *SNQ2* of *C. glabrata*. All three genes have been shown to be major determinants of azole antifungal resistance in clinical isolates (59, 87, 88, 106, 113). In an azole-resistant clinical isolate overexpressing both *CDR1* and *PDH1*, disruption of *CDR1* resulted in increased fluconazole accumulation and hypersusceptibility to fluconazole. Subsequent disruption of *PDH1* resulted in an even greater increase in azole susceptibility (87, 88). Expression of both *CDR1* and *PDH1* in a mutant of *S. cerevisiae* lacking Pdr5p resulted in increased fluconazole resistance. As with *C. albicans*, *CDR1* appears to play a more significant role in azole resistance in *C. glabrata*. Other clinical fluconazole-resistant isolates have been observed to overexpress *SNQ2*. In a resistant clinical isolate found to overexpress *SNQ2*, but not *CDR1* or *PDH1*, disruption of *SNQ2* resulted in decreased azole resistance. Overexpression of this gene in an *S. cerevisiae* mutant lacking Pdr5p resulted in increased resistance, demonstrating a role for the transporter in azole resistance in *C. glabrata* (106). *CDR1* homologs have also been identified in *C. tropicalis* and *C. krusei*, but their roles in azole resistance in clinical isolates have not been demonstrated (4, 41).

ALTERATIONS IN DRUG TARGET AS A MECHANISM OF AZOLE RESISTANCE

Mutations in the *ERG11* gene, which encodes lanosterol demethylase, the target of the azole antifungals, represent a significant mechanism of azole resistance in *C. albicans* (28, 72, 86). Such mutations presumably influence the interaction between the azole antifungal and lanosterol demethylase without significantly interfering with enzyme activity. Many unique nonsynonymous nucleotide polymorphisms have been reported for *ERG11* in azole-resistant clinical isolates of *C. albicans*, yet only a handful have been shown to directly influence azole resistance (49, 62, 86). Such mutations often occur in combination with changes to other resistance-influencing molecules, resulting in an even greater impact on azole resistance.

Overexpression of *ERG11* has also been reported for azole-resistant clinical *C. albicans* isolates (72, 83, 115). Increased expression of *ERG11* presumably results in production of more lanosterol demethylase, requiring greater amounts of azole to inhibit its activity. Moreover, overexpression of *ERG11* alleles carrying mutations conferring azole resistance likely have an additive or even synergistic effect.

TRANSCRIPTIONAL REGULATION OF *MDR1*

The *MDR1*, *CDR1*, *CDR2*, and *ERG11* genes of *C. albicans* and the *CDR1*, *PDH1*, and *ERG11* genes of *C. glabrata* have all been shown to be upregulated in response to azole exposure (35, 38, 48, 51, 52, 92, 102, 110). However, in azole-resistant isolates, these genes are constitutively activated, as they remain upregulated when grown in the absence of drug. Overexpression of *MDR1*, *CDR1*, and *CDR2* in *C. albicans* has been shown to be due to transcriptional activation, as opposed to gene amplification (56). In order to determine the cause of constitutive overexpression of *MDR1*, the promoter sequences of *MDR1* alleles of two azole-resistant *C. albicans* isolates were compared to those of their respective matched susceptible counterparts. No mutations that might be responsible for altered *MDR1* activation were observed, suggesting a role for a *trans* element in the constitutive overexpression of *MDR1* (118). When a green fluorescent protein (GFP) reporter construct using the *MDR1* promoter from an azole-susceptible strain was introduced into an azole-resistant strain that constitutively overexpresses *MDR1*, increased fluorescence was observed, demonstrating the involvement of a *trans* element in the constitutive activation of this gene. The *MDR1* promoter contains an activating protein 1 (AP-1)-like binding site (5′-TTAGTAA-3′), suggesting that a transcription factor of this family may participate in the regulation of *MDR1* expression. In *S. cerevisiae*, the bZip transcription factor *YAP1*, which is homologous to mammalian AP-1, controls the expression of genes involved in the oxidative stress response. Interestingly, the Yap1p transcription factor also controls the expression of the *FLR1* MFS transporter, which confers azole resistance on this organism (1). The homolog of *YAP1* in *C. albicans* is *CAP1*, and this gene has been shown to regulate *FLR1* expression and azole resistance when expressed in *S. cerevisiae*. Like ScYap1p, Cap1p induces expression of its target genes in response to cell stress imposed by any number of heavy metal or oxidizing agents. The two transcription factors are similarly activated through a redox-induced conformational change of the cysteine-rich domain, leading to unimpeded nuclear translocation and subsequent DNA binding and transcriptional activation. Expression of a hyperactive *CAP1* allele likewise resulted in increased expression of *MDR1* and fluconazole resistance. Disruption of *CAP1* in an azole-resistant clinical isolate overexpressing *MDR1*, however, did not reduce *MDR1* expression or fluconazole resistance (2). While *CAP1* is a regulator of *MDR1*, it does not appear to be a determinant of fluconazole resistance in clinical isolates of *C. albicans* (122).

Until recently, the molecular mechanisms leading to high-level expression of *MDR1* have been poorly understood. Recent studies indicate that three independent regions of the *MDR1* promoter contribute to *MDR1* expression in a fluconazole-resistant isolate overexpressing *MDR1* (Fig. 1). One particular region of the *MDR1* promoter (–588 to –500) mediates benomyl induction of *MDR1* transcription in the laboratory strain CAI4 (37). Work from another group using *C. albicans* laboratory strain CAI8 identified at least two *cis*-acting promoter regions that contribute to *MDR1* expression (33). In these studies, the more proximal *cis*-acting element (–399 to –299) was responsible for benomyl-induced transcription of *MDR1*, while the more distal *cis*-acting element (–601 to –500) was implicated in *MDR1* induction by oxidizing agents. The most distal region identified by both groups (–601/–588 to –500) contains a sequence that resembles and functions like the

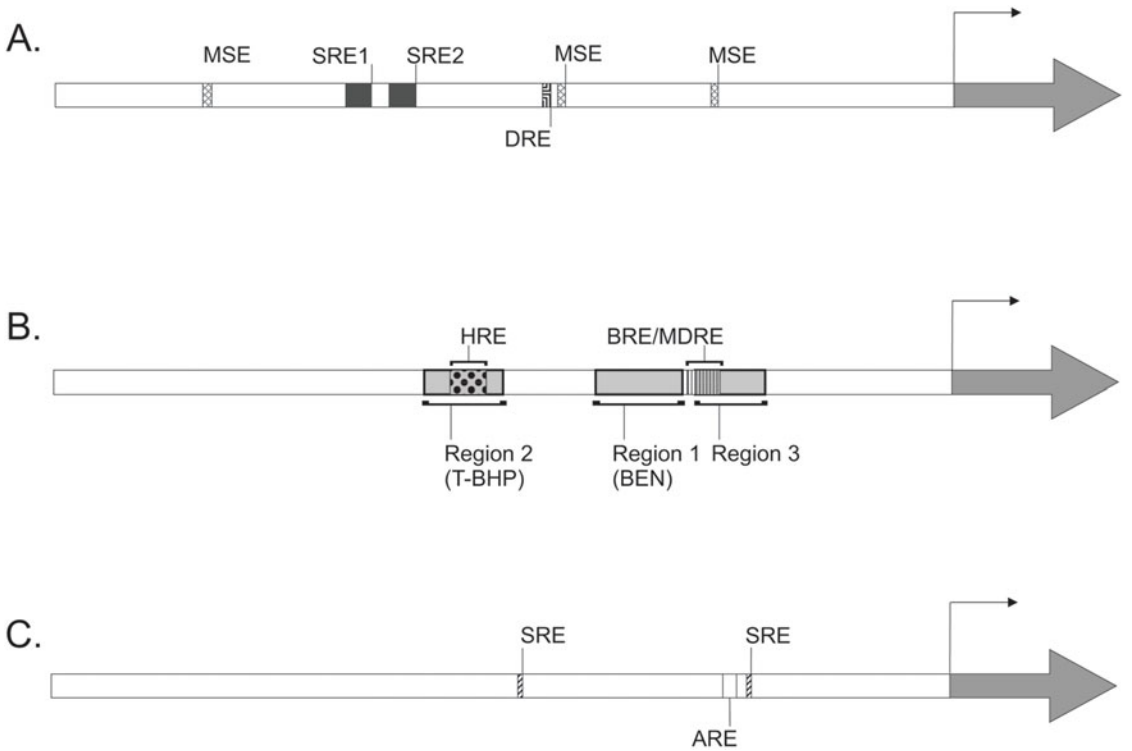

FIGURE 1 Promoter regions containing known response elements of the *CaCDR1*, *CaMDR1*, and *CaERG11* genes. Each binding region is shaded or patterned according to the type of response element, the 5′ boundary of the ORFs is indicated by the bent arrow, and the ORFs are indicated by the shaded arrow. (A) The *CDR1* gene promoter contains MSEs putatively bound by Ndt80p (11), SREs shown to be progesterone responsive (SRE1) or progesterone- and estradiol-responsive (SRE2) (40), and a DRE putatively bound by Tac1p (17, 53). (B) The *MDR1* gene promoter contains an HRE and BRE (80), which both overlap with constitutive activation regions 2 and 3, respectively (37). An additional region (region 1) was also identified by Hiller et al. (37). The MDRE, putatively bound by Mcm1p (78), is contained within the boundaries of the BRE. The *t*-butyl-hydroperoxide (T-BHP)-responsive and benomyl (BEN)-responsive elements (33) are found within regions 2 and 1, respectively. (C) The *ERG11* gene promoter contains two SREs, presumably bound by Upc2p (123), and an azole response element (ARE) (100). 10.1128/9781555817176.ch25f1

Yap1p-responsive element of *S. cerevisiae* and is the likely Cap1p-responsive element.

In a similar analysis, another group identified two *cis*-acting regulatory elements within the *MDR1* promoter that are necessary and sufficient to confer the same transcriptional responses on a heterologous promoter (80). One, which they termed the benomyl response element (BRE), is situated at position –296 to –260. It is required for both benomyl-dependent *MDR1* upregulation and constitutive high expression of *MDR1*. A second element, termed the H_2O_2 response element (HRE), is situated at position –561 to –520. The HRE is required for H_2O_2-dependent *MDR1* upregulation but dispensable for constitutive higher expression. This likely represents a refinement of the putative Cap1p-responsive element in the *MDR1* promoter described above. These investigators also defined a region that was sufficient to detect in vitro sequence-specific binding of protein complexes in crude extracts prepared from *C. albicans* (–290 to –273) within the BRE. This sequence includes a perfect match to the consensus binding sequence of Mcm1p, suggesting that *MDR1* may be a direct target of this MADS box transcriptional activator. Similar observations were made by another group, demonstrating that indeed Mcm1p participates in the regulation of *MDR1* expression (78).

The transcriptional regulator of constitutive *MDR1* expression in azole-resistant isolates was identified using genome-wide gene expression profiling analysis (63). Three matched isolate pairs of fluconazole-resistant and -susceptible clinical isolates were selected, as each of the resistant isolates overexpressed *MDR1*. The gene expression profiles of these isolates were compared in order to identify genes that are consistently coregulated with *MDR1*. One of these genes (orf19.7372) encoded a putative zinc cluster transcription factor. Disruption of this gene in clinical isolates overexpressing *MDR1* resulted in reduced resistance to fluconazole and other *MDR1* substrates, as well as reduced expression of *MDR1*. This gene was designated *MRR1* for multidrug resistance regulator. Interestingly, whereas disruption of *MDR1* partially reduces fluconazole resistance in these isolates, disruption of *MRR1* results in complete reduction of resistance, suggesting that other Mrr1p target genes may also play a role in resistance. Disruption of *MRR1* in fluconazole-susceptible isolates abolished *MDR1* induction by benomyl and hydrogen peroxide, demonstrating that this transcription factor also regulates inducible expres-

sion of *MDR1*. Examination of additional clinical isolates and laboratory-derived strains constitutively overexpressing *MDR1* have revealed that all of these contain gain-of-function mutations in *MRR1* that result in its constitutive activation (Fig. 2) (22). Likewise, in *C. dubliniensis*, constitutive upregulation of *MDR1* and associated fluconazole resistance is due to gain-of-function mutations in an orthologous gene (CdMrr1p) (93).

TRANSCRIPTIONAL REGULATION OF *CDR1* AND *CDR2*

Studies using a *Renilla* luciferase reporter system fused to *CDR1* and *CDR2* promoters cloned from azole-susceptible *C. albicans* isolates showed that their expression was increased in an azole-resistant strain in which these genes are constitutively upregulated (19). These data pointed to a *trans* element as having a direct role in constitutive overexpression of these ABC transporters and corresponding azole resistance. Investigators also identified a common unique drug response element (DRE) in the promoters of *CDR1* and *CDR2* that was required for fluphenazine and estradiol induction of these genes as well as their constitutive upregulation in azole-resistant clinical isolates (Fig. 1). The DRE contains two CGG triplets that suggested involvement of a zinc cluster transcription factor in regulating this response. In addition to the DRE, a basal expression element, a steroid-responsive element (SRE), and a negative regulatory element have all been described for *CDR1* but not *CDR2* (Fig. 1).

Among genes in the *C. albicans* genome encoding putative zinc cluster transcription factors, three were found in close proximity of the mating type locus. It had been previously noted that homozygosity of the mating type locus was associated with azole resistance in *C. albicans*. Disruption of one of these genes in an azole-susceptible strain resulted in both hypersusceptibility to several antifungal agents that are *CDR1* and *CDR2* substrates and loss of *CDR1* induction by fluphenazine (16, 17). This gene was designated *TAC1* for transcriptional activator of CDR genes. Tac1p has been shown to bind to the DRE of *CDR1* and *CDR2*. Disruption of *TAC1* in an azole-resistant clinical isolate overexpressing *CDR1* and *CDR2* abolished expression of these genes as well as azole resistance. Moreover, *TAC1* alleles carrying gain-of-function mutations result in upregulation of *CDR1* and *CDR2* as well as increased azole resistance when expressed in an otherwise azole-susceptible strain (Fig. 2). Examination of gain-of-function alleles in fluconazole-resistant *C. albicans* isolates indicates that *TAC1* alleles are codominant; therefore, *TAC1*-mediated, high-level azole resistance only occurs in cells in which there is a loss of heterozygosity resulting in the presence of only the hyperactive *TAC1*

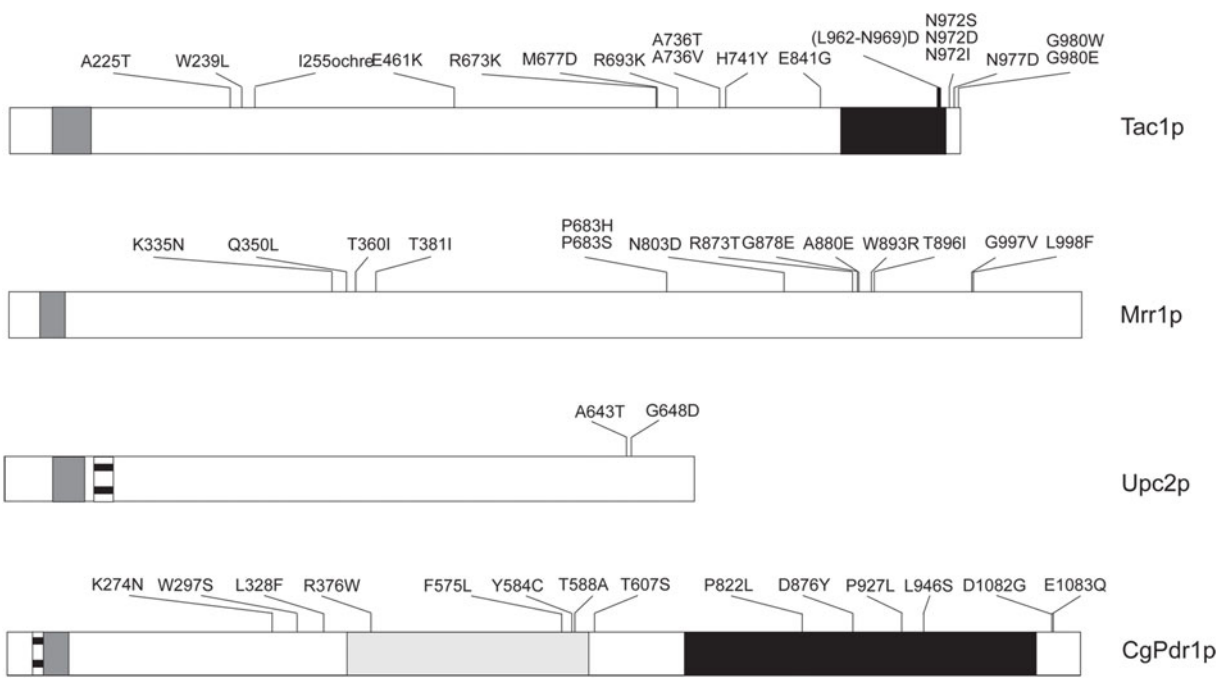

FIGURE 2 Representation of gain-of-function mutations identified to date in Tac1p, Mrr1p, Upc2p, and CgPdr1p. Each protein is displayed from N to C terminus (left to right). The shaded and patterned boxes correspond to functional motifs defined as follows: Zn_2Cys_6 zinc finger DNA-binding domain in medium gray boxes, activation domain of Tac1p (17) and of CgPdr1p (108) in black boxes, putative NLS of Upc2p and of CgPdr1p in horizontally hatched boxes, and xenobiotic binding domain of CgPdr1p (105) in light gray boxes. Only gain-of-function mutations that have been experimentally verified are shown. The Tac1p mutations were originally described by Coste et al. (16, 17) and Znaidi et al. (121), the Mrr1p mutations were originally described by Morschhäuser et al. (63) and Dunkel et al. (22), the Upc2p mutations were originally described by Dunkel et al. (23) and Heilmann et al. (34), and the Pdr1p mutations were originally described by Ferrari et al. (24), Torelli et al. (106), Tsai et al. (108), Vermitsky and Edlind (110), and K. E. Caudle et al. (unpublished data). 10.1128/9781555817176.ch25f2

allele (16). Studies indicate that this loss of heterozygosity occurs by aneuploidy, specifically a gain of all or a portion of chromosome 5, on which *TAC1* resides. Tac1p is therefore a major determinant of azole resistance in clinical isolates of *C. albicans*. Tac1p also regulates the expression of *PDR16*, which encodes a putative phosphatidylinositol transfer protein that also influences azole susceptibility (121). *PDR16* is coinduced with *CDR1* and *CDR2* in azole-resistant clinical isolates and upon fluphenazine treatment of an azole-susceptible strain. Deleting *PDR16* in an azole-resistant clinical isolate reduced resistance twofold, demonstrating that this gene contributes to the azole resistance phenotype of the cells, in addition to *CDR1* and *CDR2* (82). This raises the possibility that other targets of Tac1p may also influence clinical azole resistance.

The promoters of *CDR1*, *PDH1*, and *SNQ2* genes in *C. glabrata* all contain sequences consistent with the pleiotropic drug resistance element found in genes of *S. cerevisiae* that are regulated by the zinc cluster transcription factors Pdr1p and Pdr3p (87, 106, 111). Gain-of-function mutations in the genes encoding these transcription factors have been shown to result in constitutive upregulation of their target genes, including the *CDR1* homolog *PDR5* (68). These observations strongly suggested the existence of homologs of *PDR1* and *PDR3* that might directly affect azole resistance in *C. glabrata*. Analysis of the *C. glabrata* genome sequence (21) revealed a single *PDR1-PDR3* homolog, designated Cg*PDR1*, and a putative gain-of-function mutation in this gene was identified in azole-resistant laboratory mutant F15 (Fig. 2) (110). Expression of this mutant allele in a strain in which Cg*PDR1* had been disrupted demonstrated that this mutation was in and of itself capable of causing both increased expression of *CDR1* and *PDH1* and increased fluconazole resistance (111). Subsequently, similar gain-of-function mutations were identified in azole-resistant clinical isolates overexpressing *CDR1*, *PDH1*, and *SNQ2* (5, 24, 106). Interestingly, different gain-of-function mutations in *PDR1* appear to have different effects on the expression of *CDR1*, *PDH1*, and *SNQ2*, as mutations have been described that result in overexpression of only one of these genes (24). Moreover, clinical isolates have been described that overexpress *CDR1* and/or *CDR2* that do not appear to have a mutation in *PDR1*, suggesting alternative mechanisms for the upregulation of these genes (24).

TRANSCRIPTIONAL REGULATION OF *ERG11*

In *S. cerevisiae*, an 11-bp SRE was identified in the promoters of *ERG2* and *ERG3* and was later found in the promoter regions of other ergosterol biosynthesis genes (112). Likewise, many *C. albicans* ergosterol biosynthesis genes also contain the known *S. cerevisiae* SRE core motif (5′-TCGTATA-3′) in their promoters, suggesting a role for the transcriptional regulators that recognize and bind to this element in azole resistance (57, 100). In *S. cerevisiae*, the genes involved in the ergosterol biosynthesis pathway are regulated by the zinc cluster transcription factors Ecm22p and Upc2p, which bind to the SRE (112). A single *ECM22-UPC2* homolog has been identified in *C. albicans*, designated *UPC2*, which has been implicated as a regulator of *ERG11* expression and susceptibility to fluconazole (57, 100). Deletion of *UPC2* in a wild-type azole-susceptible strain results in hypersusceptibility to azoles, reduced accumulation of cholesterol, and abrogated expression of *ERG11* in response to azole exposure. More recently, an azole-responsive enhancer element was identified in the *ERG11* promoter and was shown to be controlled by Upc2p (Fig. 1) (69). Sequencing of *UPC2* from matched pairs of azole-resistant clinical isolates that exhibit overexpression of *ERG11* in association with fluconazole resistance and their azole-susceptible counterparts has resulted in the identification of two gain-of-function mutations that contribute to this phenotype (Fig. 2) (23, 34). Interestingly, no mutations in *UPC2* were found in other isolates overexpressing *ERG11*, suggesting alternate mechanisms for overexpression of this gene in these isolates. One such mechanism includes the aforementioned aneuploidy of chromosome 5. Both *TAC1* and *ERG11* reside on the left arm of chromosome 5, which has been shown to undergo whole-chromosome duplication or recombination to form an isochromosome, resulting in increased copy numbers of those genes (96). However, aneuploidy alone would result in only a modest increase in *ERG11* expression and is not sufficient to account for the degree of expression observed in some isolates.

OTHER TRANSCRIPTIONAL ACTIVATORS AND COACTIVATORS THAT INFLUENCE AZOLE SUSCEPTIBILITY

CaNdt80p

CaNdt80p is the homolog of the *S. cerevisiae* transcription factor ScNdt80p (11). CaNdt80p was initially identified as a regulator of *CDR1* expression, since overexpression of *NDT80* led to increased β-galactosidase activity in a reporter assay and *ndt80*/*ndt80* cells were more susceptible to fluconazole and voriconazole as measured by spot assay. While there is significant similarity between these two molecules, especially considering that the DNA-binding domain of ScNdt80p can functionally complement that of CaNdt80p, there is little homology between the activation domains of these orthologs. To compare the functions of CaNdt80p and ScNdt80p, chimeric proteins consisting of different combinations of the ScNdt80p and CaNdt80p activation and DNA-binding functional domains were introduced into a strain of *S. cerevisiae* containing a *CDR1* promoter-*lacZ* reporter construct (114). The chimeras containing the CaNdt80p activation domain and either the ScNdt80p or CaNdt80p DNA-binding domain were able to produce β-galactosidase activity over that of background. Comparisons of ScNdt80p homologs from several higher eukaryotes, including *Neurospora crassa, Caenorhabditis elegans, Drosophila melanogaster,* and humans revealed that Arg177 and Arg254 in the ScNdt80p DNA-binding domain are absolutely conserved. Therefore, in order to test whether either of these residues is necessary for transcriptional activation through the DNA-binding domain, the Arg432 residue, which corresponds to the ScNdt80p Arg177 residue, was mutated to an alanine. This mutated *CaNDT80*^{R432A} failed to complement *ndt80*Δ/*ndt80*Δ *C. albicans* cells, indicating that the conserved arginine is required for transactivating Ndt80p targets.

To generate a more global assessment of Ndt80p, Sellam et al. (94) performed genome-wide location analysis. Chromatin immunoprecipitation-on-a-chip (ChIP-chip) hybridization and subsequent gene ontology term gene categorization revealed a significant enrichment of genes involved in the cell wall, carbohydrate metabolism, response to heat stress, response to osmotic stress, alcohol metabolism, cell adhesion, cell cycle, glycolysis, and hyphal growth. In addition, a portion of the genes identified were transcription factors whose target genes are involved with the aforemen-

tioned cellular processes. More pertinent to the scope of this chapter, Ndt80p bound to the promoters of many genes previously identified as being associated with azole resistance, including the ABC transporter genes *CDR1*, *CDR2*, and *CDR4*; the MFS transporter genes *MDR1*, *FLU1*, *NAG3*, and *NAG4*; the flippase genes *RTA2* and *RTA3*; and other azole resistance-associated genes, *PDR16*, *ERG3*, and *ERG11*. It should be noted that no transcription factors previously associated with any of these resistance-associated genes (i.e., *TAC1*, *UPC2*, or *MRR1*) were identified as being Ndt80p targets in this analysis, suggesting that Ndt80p may provide an additional level of transcriptional control for these resistance genes. The authors then compared the gene expression profiles of fluconazole-treated wild-type and *ndt80*/*ndt80* null mutant cells to determine which genes' induction is dependent upon Ndt80p. While seven ergosterol biosynthesis genes and *UPC2* were induced in wild-type cells upon azole treatment as previously shown by Liu et al. (52), they were not induced to the same extent in fluconazole-treated *ndt80*/*ndt80* cells.

CaMcm1p

As noted previously, prior to the discovery of Mrr1p as the transcriptional regulator responsible for regulating Mdr1p-mediated azole resistance in *C. albicans*, studies were undertaken to determine which factors are involved in regulating *MDR1*. Investigators defined regions of the *MDR1* promoter that are necessary for *MDR1* induction and subsequently determined which factors could bind these promoter elements. One such region was the *MDR1* drug resistance element (MDRE), required for *MDR1* overexpression in azole-resistant strains (78). Identification of the MDRE was accomplished by testing laboratory-derived azole-resistant strains for their ability to activate transcription of GFP from an *MDR1* promoter or *CDR1* promoter reporter plasmid. Five of the 12 strains tested exhibited increased *MDR1* promoter activity and also possessed increased *MDR1* gene expression as measured by Northern hybridization. Subsequent testing of various smaller regions of the *MDR1* promoter in reporter assays led to the determination that the MDRE is found between positions −295 and −261 relative to the *MDR1* initiation codon. When the MDRE was cloned in a GFP reporter construct containing a nonfunctional promoter, it was able to activate transcription in a dose-dependent manner. The authors recognized that a portion of the MDRE was actually an inverted repeat that comprised a previously defined Mcm1p binding site in *S. cerevisiae*.

To demonstrate specific physical interaction of a factor or factors with the MDRE, whole-cell protein extracts from fluconazole-susceptible and fluconazole-resistant strains were used in an electrophoretic mobility shift assay (EMSA). Both cell extracts contained a protein or proteins that bound an oligonucleotide containing the MDRE, and this interaction could be competed by the addition of a 100-fold excess of unlabeled MDRE-containing oligonucleotide but not by a 100-fold excess of unlabeled irrelevant oligonucleotide. In addition, when equal amounts of protein from fluconazole-susceptible and fluconazole-resistant cell extracts were used in an EMSA, there was a fivefold enrichment in the shifted MDRE oligonucleotide-protein complex in the fluconazole-resistant sample. The portion of the MDRE required for factor binding was found to be between positions −288 and −275 (relative to the beginning of the *MDR1* open reading frame [ORF]) of the MDRE, as determined by use of a series of oligonucleotides containing mutated MDREs in oligonucleotide competition EMSAs. This discrete sequence matched the ScMcm1p binding sequence. Assuming that this sequence in *C. albicans* could also be bound by the *C. albicans* ortholog of ScMcm1p, whole-cell extracts from a CaMcm1p-depleted *C. albicans* strain were used in an EMSA with the MDRE oligonucleotides. While the MDRE-specific shifted band was present in the non-CaMcm1p-depleted cell extracts, this band was undetectable using CaMcm1p-depleted cell extracts. This conclusively showed that Mcm1p binds to the MDRE in *C. albicans*.

Mcm1p is a MADS box cell cycle regulator that plays a critical role in regulating the prereplication complex in *S. cerevisiae*. In determining the power of utilizing an array of molecular tags for tagging a DNA-binding protein and performing subsequent genome-wide location analyses, Lavoie et al. (50) used a tandem affinity purification (TAP) tag approach to enrich DNA fragments with which CaMcm1p specifically associates. They then identified the relative location of these DNA fragments within the *C. albicans* genome via ChIP-chip. The ChIP-chip analysis demonstrated that CaMcm1p binds promoter regions of several genes that are involved in arginine metabolism, suggesting that CaMcm1p plays the roles of both ScMcm1p and ScArg80p, which are paralogous transcription factors. Not surprisingly, CaMcm1p also binds the promoter regions of several genes encoding proteins comprising the Mcm complex. Finally, the authors measured a 7.49-fold enrichment of CaMcm1p binding of the *MDR1* promoter, providing independent confirmation that CaMcm1p does associate with a region of the *MDR1* promoter. Although this is a significant finding, further studies are needed to determine the nature of this association and how CaMcm1p mechanistically affects the transcription of *MDR1*, especially in azole-resistant *C. albicans* isolates.

CaRep1p

Rep1, regulator of efflux pump 1, was identified through a screening of *C. albicans* genomic DNA fragments cloned into a high-copy-number *S. cerevisiae* expression plasmid, which, upon introduction into a strain of *S. cerevisiae*, was able to enhance fluconazole susceptibility (12). Since *REP1* was the only full-length ORF on a 2.7-kb fragment that yielded the desired phenotype, this change in fluconazole sensitivity was wholly attributable to Rep1. Sequence analysis indicated that Rep1p has a DNA-binding domain similar to that of CaNdt80p and ScNdt80p, both which modulate target gene expression through binding a mid-sporulation element (MSE) found in the promoter region of some genes.

Null mutant strains of *REP1* had reduced susceptibility to fluconazole, indicating that Rep1p acts as a negative regulator of a gene or genes required for conferring azole resistance. In order to investigate which molecules are regulated by Rep1p, wild-type and *rep1*Δ/*rep1*Δ *C. albicans* cells were treated with miconazole (an inducer of *CDR1* gene expression) or 4-nitroquinoline 1-oxide (4-NQO; an inducer of *MDR1* gene expression), and *MDR1* and *CDR1* gene expression was examined in these cells by real-time reverse transcription-PCR. There was no change in *CDR1* gene induction levels in the miconazole-treated *rep1*Δ/*rep1*Δ mutant strain; however, *MDR1* gene expression was enhanced 10-fold in 4-NQO-treated *rep1*Δ/*rep1*Δ cells compared to 4-NQO-treated wild-type cells. A decrease in both fluconazole and voriconazole susceptibility in *rep1*Δ/*rep1*Δ mutant cells was observed.

Although it is still unclear how Rep1p affects *MDR1* gene expression, Chen et al. demonstrated that Rep1p does not affect expression of *MRR1* or *CAP1*, two transcription factors known to control *MDR1* gene expression (2, 12, 63). The authors surmised that either Rep1p could bind Mrr1p directly to repress Mrr1p-mediated transcription in the absence of inducers or Rep1p could occupy MSEs in the target gene promoter, which would interfere somehow with Mrr1p promoter binding. Interestingly, when various null mutants were examined for the ability of 4-NQO to induce *MDR1* gene expression, *MDR1* expression was ablated in the *mrr1*Δ/*mrr1*Δ strain and was induced to a greater extent in the *rep1*Δ/*rep1*Δ strain than in the wild-type strain. However, when *MDR1* gene expression was examined in an *mrr1*Δ/*mrr1*Δ *rep1*Δ/*rep1*Δ strain, the level was nearly identical to that of wild-type cells. These data suggest that because *MDR1* expression is greater in *rep1*Δ/*rep1*Δ cells, Rep1p acts as a repressor of Mrr1p-mediated *MDR1* expression in response to 4-NQO. In the absence of both *REP1* and *MRR1*, *MDR1* expression is the same as in wild-type cells, suggesting that another factor is able to induce in the absence of *MRR1* and *REP1*. The authors hypothesized that this factor could be Ndt80p, since it has a type of DNA-binding domain similar to that of Rep1p and could in theory occupy the same MSEs in *rep1*Δ/*rep1*Δ *mrr1*Δ/*mrr1*Δ cells to induce *MDR1* gene expression. Although Ndt80p has recently been shown to occupy the promoter of *MDR1* (94), it is unclear whether Ndt80p is responsive to 4-NQO or whether it can induce expression of *MDR1*. Another possibility is that, assuming that Rep1p binds Mrr1p directly, Rep1 could also bind other transcription factors such as Cap1p, which could then induce *MDR1* expression in the absence of Rep1p. Clearly, further studies are warranted to fully elucidate the mechanism by which Rep1p affects transcription.

CaAda2p

Given that exposure to antifungal compounds such as azoles can elicit cellular stress responses, studies have been undertaken to determine whether stress response genes may play a role in azole resistance. The Spt-Ada-Gcn5-acetyltransferase (SAGA) coactivator complex has been widely studied for its involvement in remodeling chromatin, especially in stress-responsive gene promoters. Among the activation proteins (Ada1 to Ada5) that are components of the SAGA coactivator complex, Ada2p interacts directly with the activation domains of Gcn4p and Gal4p and indirectly with TATA-binding protein. Consequently, Ada2p may direct the interactions between certain transcription factors and components of the transcriptional machinery.

In *C. albicans*, Ada2p was found to be required for the cell wall damage response elicited by caspofungin (7). It was speculated that Ada2p would be recruited by the transcription factor Cas5p to coactivate a cell wall integrity stress response. Genome-wide location analysis revealed that Ada2p binds in the promoter region of 200 ORFs in *C. albicans* (95). Among the most enriched gene ontology term categories of Ada2p-bound promoter genes were the response to oxidative stress and response to drug. Not surprisingly, *ada2*Δ/*ada2*Δ cells were more sensitive to growth in fluconazole, menadione, and hydrogen peroxide. This observation led to an examination of expression of genes known to be involved in oxidative stress response and drug response. The ability of menadione to induce expression of the *CAP1*, *TRX1*, *GPX2*, *SOD6*, and *HSF1* genes was impaired in *ada2*Δ/*ada2*Δ cells, which is consistent with the involvement of Ada2p in oxidative stress response. Similarly, *CDR1*, *PDR16*, and *MDR1* gene expression was significantly lower in fluconazole-treated *ada2*Δ/*ada2*Δ cells than in treated wild-type cells. It should be noted that *NDT80* gene expression levels were not affected in the *ada2*Δ/*ada2*Δ cells, suggesting that the effect of Ada2p on *CDR1* gene expression did not involve control of *NDT80* gene expression.

When the authors compared the Ada2p-bound promoters with those of Cap1-dependent genes (genes whose expression is altered in an H_2O_2-treated *cap1*Δ/*cap1*Δ mutant strain), they found that there were 15 common genes. When a TAP-tagged Ada2p was mapped for its ability to occupy the promoter region 200 bp immediately upstream of each of these ORFs in a *cap1*Δ/*cap1*Δ mutant strain, *GRP2*, *GPX2*, *TTR1*, *YCF1*, *MDR1*, and *CIP1* were significantly affected. This demonstrated that Ada2p is recruited to the promoters of these Cap1p target genes. Similarly, comparison of the Ada2p-bound promoters, constitutively activated genes from *MDR1*-overexpressing, azole-resistant *C. albicans* isolates, and genes specifically regulated by an *MRR1* allele harboring a gain-of-function mutation yielded nine genes, including *IFD1*, *CSH1*, *HSP31*, *IPF5987*, *GRP2*, *MDR1*, *OYE32*, *GPX1*, and *RPL30*. The ability of Ada2p to bind the 200-bp region immediately upstream of any of these orfs in an *mrr1*Δ/*mrr1*Δ mutant strain was significantly affected for six genes: *IFD1*, *CSH1*, *HSP31*, *IPF5987*, *GRP2*, and *MDR1*. These data demonstrate that Ada2p acts as a coactivator for Mrr1p for these genes. However, these authors mention that disruption of *ADA2* in an *MDR1*-overexpressing, azole-resistant isolate did not alter fluconazole sensitivity, suggesting either a redundancy of coactivators, the possibility that the *MRR1* gain-of-function allele no longer requires a coactivator, or the involvement of Ada2p in azole tolerance rather than azole resistance.

CaCrm1p

Crm1p, also known as exportin, is a chaperone of proteins that contain a leucine-rich nuclear export signal (NES); this molecule is well conserved evolutionarily, as orthologs of Crm1p are found in humans, *Drosophila melanogaster*, *Caenorhabditis elegans*, and yeasts. In yeasts, two AP-1-like transcription factors, Yap1p in *S. cerevisiae* and Pap1p in the fission yeast *Schizosaccharomyces pombe*, are known targets of Crm1p. In cells not subjected to oxidative stress, Crm1p is bound to the NES of these AP-1 transcription factors to inhibit translocation of Yap1p and Pap1p into the nucleus. Treatment of cells with compounds that induce oxidative stress leads to release of the transcription factors from Crm1p, accumulation of Yap1p and Pap1p in the nucleus, and subsequent induction of target gene transcription. Raymond et al. (77) identified the *CRM1* gene in *C. albicans*. Not only is *CaCRM1* 65% identical to *ScCRM1*, but CaCrm1p was able to complement a temperature-sensitive *xpo1-1* defect in *S. cerevisiae*. Further, mutations or truncation of the cysteine-rich domain which harbors the NES in Cap1p result in constitutive localization of Cap1p in the nucleus, enhanced growth in the presence of a variety of toxic compounds, such as fluconazole, cerulenin, brefeldin A, cadmium salts, 4-NQO, and 1,10-phenanthroline, and constitutive overexpression of Cap1p target genes such as *MDR1* (2, 120).

CaCrz1p

The calcineurin pathway in *C. albicans* is involved in a variety of stress responses such as tolerance to azole antifungal compounds. Calcineurin, a Ca^{2+}-calmodulin-dependent

serine-threonine phosphatase which consists of catalytic subunit (Cna) and regulatory subunit (Cnb1), becomes activated during release of intracellular calcium brought upon by extracellular cues. Once activated via conformational changes due to interaction with Ca^{2+}-bound calmodulin, calcineurin is able to regulate downstream target molecules through its phosphatase activity. One target is Crz1p, a Cys_2His_2 zinc finger transcription factor which is sequestered in the cytoplasm while phosphorylated. Upon dephosphorylation by calcineurin, Crz1p translocates to the nucleus and activates transcription of its target genes.

The *C. albicans CRZ1* homolog was identified through BLAST searches querying *ScCRZ1* in the *Candida* Genome Database (http://www.candidagenome.org, 39, 70). Phenotypic analysis indicated that *crz1*Δ/*crz1*Δ *C. albicans* cells were more sensitive than wild-type cells to the presence of calcium, lithium, and manganese ions and 0.02% sodium dodecyl sulfate, cellular stresses known to induce the calcineurin pathway in *S. cerevisiae*. The reintroduction of *CaCRZ1* was able to restore growth in response to these stresses to wild-type levels. In contrast, there was not increased sensitivity to 5 μg of fluconazole/ml or 10 μg of terbinafine/ml in the absence of *CaCRZ1*. It should be noted that *cna*Δ/*cna*Δ cells were unable to grow in the presence of any of the stressors, including fluconazole and terbinafine. Similar analysis of *crz2*Δ/*crz2*Δ and *crz1*Δ/*crz1*Δ *crz2*Δ/*crz2*Δ strains indicated that although the *CRZ2* gene shares significant sequence homology to *CRZ1*, *CRZ2* is not a target of calcineurin, as the absence of *CRZ2* in each strain did not change its growth phenotype in the presence of stressors. *CRZ1* also affected the ability of cells to form filaments on spider medium, an observation previously made for the *cna*/*cna* strain (90); however, the *crz1*Δ/*crz1*Δ strain was able to grow on agar containing serum, albeit without filamentation, whereas *cna*Δ/*cna*Δ cells were unable to grow at all in the presence of serum.

CaCrz1p was demonstrated to translocate to the nucleus upon treatment of cells with calcium, and this was regulated in a calcineurin-dependent manner, since the addition of the calcineur inhibitor FK506 led to sequestration of Crz1p in the cytoplasm even with the addition of calcium. When a construct of *CRZ1* lacking the putative serine-rich region (SRR) was introduced into *crz1*Δ/*crz1*Δ cells, Crz1p was constitutively present in the nucleus, indicating that the SRR was necessary for calcineurin-dependent regulation and was thus the region harboring phosphorylated/dephosphorylated residues. However, the addition of FK506 abolishes nuclear localization of this Crz1p mutant, even in the presence of calcium.

Partial-deletion constructs of Crz1p were tested in a similar fashion to determine the location of the nuclear localization signal (NLS) and NES, two fundamental regulatory regions that are exposed alternately due to the conformational changes dictated by the calcineurin-dependent phosphorylation state of the protein. An N-terminally truncated Crz1p was constitutively translocated in the cytoplasm even with calcium treatment, indicating that the NLS is most likely in the N-terminal portion of Crz1p. Alternatively, a C-terminally truncated Crz1p as well as a Crz1p mutant lacking 90 residues in the middle of the protein were both constitutively present in the nucleus. These data suggest that the NLS resides in the N-terminal portion of the protein between the initial methionine residue and the beginning of the SRR at residue 207. Genome-wide expression analysis revealed 65 genes activated by Crz1p in the presence of calcium. Of these genes, *DDR48*, *GRP2*, and *RTA2* had previously been associated with azole resistance in *C. albicans* (79).

Recently, the role of calcineurin and Crz1p was examined in *C. glabrata* (60). The *CNB1* and *CRZ1* genes were singularly disrupted in a wild-type strain of *C. glabrata* and fluconazole MICs were assessed. Whereas the *crz1*Δ strain had an increased fluconazole MIC compared to that of the wild type (32 and 16 μg/ml, respectively), the *cnb1*Δ strain had a lower fluconazole MIC (4 μg/ml). The fluconazole MICs returned to 16 μg/ml in each of the reconstituted strains. The authors concluded that calcineurin is involved in azole tolerance by a Crz1-independent mechanism.

CaFcr3p and CaFcr1p

Like Cap1p, Fcr3p is an AP-1-like transcription factor in *C. albicans* that was identified for its ability to confer decreased susceptibility on a hypersusceptible *pdr1*Δ/*pdr3*Δ strain of *S. cerevisiae* (119). Sequence analysis indicated that the *FCR3* gene is closely related to *ScYAP3* (34% identical). It is unclear how Fcr3p is activated in order to induce transcription of target genes, and Fcr3p target genes in *C. albicans* are unknown. The transcription factor was able to activate transcription from the *ScPDR5* promoter, as measured by β-galactosidase assay, and was able to induce *ScPDR5* gene expression in a *pdr1*Δ/*pdr3*Δ null strain of *S. cerevisiae* (104, 119). In addition, *FCR3* conferred reduced hypersusceptibility to 4-NQO (which is not a substrate for Pdr5p) in a *pdr1*Δ/*pdr3*Δ *S. cerevisiae* strain, indicating a role for *FCR3* in controlling expression of other drug resistance genes. Fcr1p was another transcription factor identified during the same screening of fluconazole-treated *pdr1*Δ/*pdr3*Δ *S. cerevisiae* cells (104). Sequence analysis reveals that Fcr1p is a Zn_2Cys_6 binuclear cluster transcription factor and is related to ScPdr1p. However, while disruption of *PDR1* and *PDR3* in *S. cerevisiae* results in fluconazole hypersusceptibility, disruption of both *FCR1* alleles in *C. albicans* results in an increased ability of the cells to grow in the presence of fluconazole, indicating that *FCR1* functions as a negative determinant of fluconazole resistance.

Pdr1p/Pdr3p and Gal11p

The observation that multidrug resistance in fungi often involves the overexpression of various ABC and MFS drug efflux pumps and that regulation of ABC transporter genes in mammalian cells is mediated through the pregnane X receptor (PXR) nuclear receptor led to studies to identify an analogous mechanism in yeast (105). In response to azole treatment, *S. cerevisiae* Pdr1/Pdr3p can induce expression of the gene encoding the ABC transporter Pdr5p, an ortholog to CaCdr1p. Investigators demonstrated that ScPdr1p binds [^{3}H]ketoconazole with a dissociation constant of 39 μM, similar to that of mammalian PXR, and binding of [^{3}H]ketoconazole can be completely competed with a 1,000-fold excess of unlabeled xenobiotics such as cycloheximide, rifampin, and ketoconazole itself. They then examined whether Pdr1p/Pdr3p required a cofactor for ligand-dependent gene activation of target genes. The Mediator coactivator complex is involved in transcriptional activation in a wide array of different organisms, from yeasts to humans. Of the twelve Mediator subunit-deficient *S. cerevisiae* strains screened for the ability to grow on ketoconazole, only the *gal11*Δ strain was sensitive to ketoconazole. In addition, the *gal11*Δ strain was unable to induce expression of *PDR5* in response to cycloheximide, rifampin, or ketoconazole. Myc-tagged Pdr1p (Myc_6-Pdr1p) and Flag antigen-tagged Gal11p (Gal11p-$Flag_2$) proteins were shown to coimmunoprecipi-

tate with each other; however, when the putative activator-binding KIX domain was deleted from Gal11p-$Flag_2$, Myc_6-Pdr1p could no longer associate with Gal11p. It was therefore not surprising that a strain containing the Gal11pΔKIX protein was unable to induce *PDR5* gene expression in response to cycloheximide, rifampin, or ketoconazole and was subsequently sensitive to growth in ketoconazole.

In the same study, the *C. glabrata* single-gene ortholog of Pdr1p/Pdr3p, CgPdr1p, was shown to complement xenobiotic-responsive *ScPDR5* gene induction in a *pdr1*Δ/*pdr3*Δ *S. cerevisiae* strain. CgPdr1p was also able to bind directly to [^{3}H]ketoconazole, indicating PXR activity for CgPdr1p. Therefore, *C. glabrata* genes with significant sequence homology to *ScGAL11* were pursued, and two genes named *CgGAL11A* and *CgGAL11B* were identified. Xenobiotic-dependent induction of *CgCDR2* gene expression was severely impaired in a *gal11a*Δ *C. glabrata* strain, while such induction was not affected in a *gal11b*Δ *C. glabrata* strain. Similarly, the *gal11a*Δ strain was significantly more sensitive to growth on ketoconazole plates, the *gal11b*Δ strain was not sensitive to ketoconazole, and the *gal11a*Δ/*gal11b*Δ strain behaved similarly to the *gal11a*Δ strain. Furthermore, a CgPdr1p activation domain fragment was able to associate with the CgGal11Ap KIX domain to a greater extent than with the CgGal11Bp KIX domain. These data indicate that the CgGal11Ap molecule is far more important in CgPdr1p xenobiotic-dependent gene activation than CgGal11Bp. These studies clearly illustrate the critical role that Gal11p plays in azole response in *C. glabrata*.

CONCLUSION

Much has been learned in recent years regarding the transcriptional regulation of azole antifungal resistance in *Candida*. The identification of key transcription factors that regulate azole resistance, as well as proteins that interact with these regulators and participate in this process, may lead to novel therapeutic strategies to overcome resistance or prevent its development in clinical isolates. Moreover, knowledge about mutations leading to resistance through activation of these transcription factors as well as mutations in the gene encoding the azole target Erg11p may lead to improved methodologies for predicting azole treatment failure in patients with *Candida* infections. New technologies allowing genome-wide analysis at multiple levels will likely yield new insight into the regulation of antifungal resistance and hold great promise for moving the field forward in the coming years.

We are grateful to Joachim Morschhäuser and Martine Raymond for critical reading of the manuscript. Work in our laboratory is supported by National Institutes of Health grant AI058145.

REFERENCES

1. **Alarco, A. M., I. Balan, D. Talibi, N. Mainville, and M. Raymond.** 1997. AP1-mediated multidrug resistance in *Saccharomyces cerevisiae* requires *FLR1* encoding a transporter of the major facilitator superfamily. *J. Biol. Chem.* **272:**19304–19313.
2. **Alarco, A. M., and M. Raymond.** 1999. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. *J. Bacteriol.* **181:**700–708.
3. **Balan, I., A. M. Alarco, and M. Raymond.** 1997. The *Candida albicans CDR3* gene codes for an opaque-phase ABC transporter. *J. Bacteriol.* **179:**7210–7218.
4. **Barchiesi, F., D. Calabrese, D. Sanglard, L. Falconi Di Francesco, F. Caselli, D. Giannini, A. Giacometti, S. Gavaudan, and G. Scalise.** 2000. Experimental induction of fluconazole resistance in *Candida tropicalis* ATCC 750. *Antimicrob. Agents Chemother.* **44:**1578–1584.
5. **Bennett, J. E., K. Izumikawa, and K. A. Marr.** 2004. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. *Antimicrob. Agents Chemother.* **48:**1773–1777.
6. **Ben-Yaacov, R., S. Knoller, G. A. Caldwell, J. M. Becker, and Y. Koltin.** 1994. *Candida albicans* gene encoding resistance to benomyl and methotrexate is a multidrug resistance gene. *Antimicrob. Agents Chemother.* **38:**648–652.
7. **Bruno, V. M., S. Kalachikov, R. Subaran, C. J. Nobile, C. Kyratsous, and A. P. Mitchell.** 2006. Control of the *C. albicans* cell wall damage response by transcriptional regulator Cas5. *PLoS Pathog.* **2:**e21.
8. **Bustamante, C. I.** 2005. Treatment of *Candida* infection: a view from the trenches! *Curr. Opin. Infect. Dis.* **18:** 490–495.
9. **Calabrese, D., J. Bille, and D. Sanglard.** 2000. A novel multidrug efflux transporter gene of the major facilitator superfamily from *Candida albicans* (*FLU1*) conferring resistance to fluconazole. *Microbiology* **146:**2743–2754.
10. **Chapeland-Leclerc, F., C. Hennequin, N. Papon, T. Noël, A. Girard, G. Socié, P. Ribaud, and C. Lacroix.** 2010. Acquisition of flucytosine, azole, and caspofungin resistance in *Candida glabrata* bloodstream isolates serially obtained from a hematopoietic stem cell transplant recipient. *Antimicrob. Agents Chemother.* **54:**1360–1362.
11. **Chen, C. G., Y. L. Yang, H. I. Shih, C. L. Su, and H. J. Lo.** 2004. CaNdt80 is involved in drug resistance in *Candida albicans* by regulating *CDR1*. *Antimicrob. Agents Chemother.* **48:**4505–4512.
12. **Chen, C. G., Y. L. Yang, K. Y. Tseng, H. I. Shih, C. H. Liou, C. C. Lin, and H. J. Lo.** 2009. Rep1p negatively regulating *MDR1* efflux pump involved in drug resistance in *Candida albicans*. *Fungal Genet. Biol.* **46:**714–720.
13. **Chen, K. H., T. Miyazaki, H. F. Tsai, and J. E. Bennett.** 2007. The bZip transcription factor Cgap1p is involved in multidrug resistance and required for activation of multidrug transporter gene *CgFLR1* in *Candida glabrata*. *Gene* **386:**63–72.
14. Reference deleted.
15. **Cleary, J. D., G. Garcia-Effron, S. W. Chapman, and D. S. Perlin.** 2008. Reduced *Candida glabrata* susceptibility secondary to an *FKS1* mutation developed during candidemia treatment. *Antimicrob. Agents Chemother.* **52:** 2263–2265.
16. **Coste, A., V. Turner, F. Ischer, J. Morschhäuser, A. Forche, A. Selmecki, J. Berman, J. Bille, and D. Sanglard.** 2006. A mutation in Tac1p, a transcription factor regulating *CDR1* and *CDR2*, is coupled with loss of heterozygosity at chromosome 5 to mediate antifungal resistance in *Candida albicans*. *Genetics* **172:**2139–2156.
17. **Coste, A. T., M. Karababa, F. Ischer, J. Bille, and D. Sanglard.** 2004. *TAC1*, transcriptional activator of *CDR* genes, is a new transcription factor involved in the regulation of *Candida albicans* ABC transporters *CDR1* and *CDR2*. *Eukaryot. Cell* **3:**1639–1652.
18. Reference deleted.
19. **de Micheli, M., J. Bille, C. Schueller, and D. Sanglard.** 2002. A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters *CDR1* and *CDR2*, two genes involved in antifungal drug resistance. *Mol. Microbiol.* **43:**1197–1214.
20. **Dizbay, M., I. Fidan, A. Kalkanci, N. Sari, B. Yalcin, S. Kustimur, and D. Arman.** 2010. High incidence of *Candida parapsilosis* candidaemia in non-neutropenic critically

ill patients: epidemiology and antifungal susceptibility. *Scand. J. Infect. Dis.* **42:**114–120.

21. **Dujon, B., D. Sherman, G. Fischer, P. Durrens, S. Casaregola, I. Lafontaine, J. De Montigny, C. Marck, C. Neuvéglise, E. Talla, N. Goffard, L. Frangeul, M. Aigle, V. Anthouard, A. Babour, V. Barbe, S. Barnay, S. Blanchin, J. M. Beckerich, E. Beyne, C. Bleykasten, A. Boisramé, J. Boyer, L. Cattolico, F. Confanioleri, A. De Daruvar, L. Despons, E. Fabre, C. Fairhead, H. Ferry-Dumazet, A. Groppi, F. Hantraye, C. Hennequin, N. Jauniaux, P. Joyet, R. Kachouri, A. Kerrest, R. Koszul, M. Lemaire, I. Lesur, L. Ma, H. Muller, J. M. Nicaud, M. Nikolski, S. Oztas, O. Ozier-Kalogeropoulos, S. Pellenz, S. Potier, G. F. Richard, M. L. Straub, A. Suleau, D. Swennen, F. Tekaia, M. Wésolowski-Louvel, E. Westhof, B. Wirth, M. Zeniou-Meyer, I. Zivanovic, M. Bolotin-Fukuhara, A. Thierry, C. Bouchier, B. Caudron, C. Scarpelli, C. Gaillardin, J. Weissenbach, P. Wincker, and J. L. Souciet.** 2004. Genome evolution in yeasts. *Nature* **430:**35–44.
22. **Dunkel, N., J. Blass, P. D. Rogers, and J. Morschhäuser.** 2008. Mutations in the multi-drug resistance regulator *MRR1*, followed by loss of heterozygosity, are the main cause of *MDR1* overexpression in fluconazole-resistant *Candida albicans* strains. *Mol. Microbiol.* **69:**827–840.
23. **Dunkel, N., T. T. Liu, K. S. Barker, R. Homayouni, J. Morschhäuser, and P. D. Rogers.** 2008. A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate. *Eukaryot. Cell* **7:**1180–1190.
24. **Ferrari, S., F. Ischer, D. Calabrese, B. Posteraro, M. Sanguinetti, G. Fadda, B. Rohde, C. Bauser, O. Bader, and D. Sanglard.** 2009. Gain of function mutations in *CgPDR1* of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. *PLoS Pathog.* **5:**e1000268.
25. **Fling, M. E., J. Kopf, A. Tamarkin, J. A. Gorman, H. A. Smith, and Y. Koltin.** 1991. Analysis of a *Candida albicans* gene that encodes a novel mechanism for resistance to benomyl and methotrexate. *Mol. Gen. Genet.* **227:**318–329.
26. **Franz, R., S. L. Kelly, D. C. Lamb, D. E. Kelly, M. Ruhnke, and J. Morschhäuser.** 1998. Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical *Candida albicans* strains. *Antimicrob. Agents Chemother.* **42:**3065–3072.
27. **Franz, R., S. Michel, and J. Morschhäuser.** 1998. A fourth gene from the *Candida albicans* CDR family of ABC transporters. *Gene* **220:**91–98.
28. **Franz, R., M. Ruhnke, and J. Morschhäuser.** 1999. Molecular aspects of fluconazole resistance development in *Candida albicans*. *Mycoses* **42:**453–458.
29. **Gaitan Cepeda, L. A., A. Ceballos Salobreña, K. López Ortega, N. Arzate Mora, and Y. Jiménez Soriano.** 2008. Oral lesions and immune reconstitution syndrome in HIV+/AIDS patients receiving highly active antiretroviral therapy. Epidemiological evidence. *Med. Oral Patol. Oral Cir. Bucal.* **13:**E85–E93.
30. **Gallis, H. A., R. H. Drew, and W. W. Pickard.** 1990. Amphotericin B: 30 years of clinical experience. *Rev. Infect. Dis.* **12:**308–329.
31. **Garcia-Effron, G., D. P. Kontoyiannis, R. E. Lewis, and D. S. Perlin.** 2008. Caspofungin-resistant *Candida tropicalis* strains causing breakthrough fungemia in patients at high risk for hematologic malignancies. *Antimicrob. Agents Chemother.* **52:**4181–4183.
32. **Ghannoum, M. A., and L. B. Rice.** 1999. Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. *Clin. Microbiol. Rev.* **12:**501–517.
33. **Harry, J. B., B. G. Oliver, J. L. Song, P. M. Silver, J. T. Little, J. Choiniere, and T. C. White.** 2005. Drug-induced regulation of the *MDR1* promoter in *Candida albicans*. *Antimicrob. Agents Chemother.* **49:**2785–2792.
34. **Heilmann, C. J., S. Schneider, K. S. Barker, P. D. Rogers, and J. Morschhäuser.** 2010. An A643T mutation in the transcription factor Upc2p causes constitutive *ERG11* upregulation and increased fluconazole resistance in *Candida albicans*. *Antimicrob. Agents Chemother.* **54:**353–359.
35. **Hernáez, M. L., C. Gil, J. Pla, and C. Nombela.** 1998. Induced expression of the *Candida albicans* multidrug resistance gene *CDR1* in response to fluconazole and other antifungals. *Yeast* **14:**517–526.
36. **Hiller, D., D. Sanglard, and J. Morschhäuser.** 2006. Overexpression of the *MDR1* gene is sufficient to confer increased resistance to toxic compounds in *Candida albicans*. *Antimicrob. Agents Chemother.* **50:**1365–1371.
37. **Hiller, D., S. Stahl, and J. Morschhäuser.** 2006. Multiple *cis*-acting sequences mediate upregulation of the *MDR1* efflux pump in a fluconazole-resistant clinical *Candida albicans* isolate. *Antimicrob. Agents Chemother.* **50:**2300–2308.
38. **Izumikawa, K., H. Kakeya, H. F. Tsai, B. Grimberg, and J. E. Bennett.** 2003. Function of *Candida glabrata* ABC transporter gene, *PDH1*. *Yeast* **20:**249–261.
39. **Karababa, M., E. Valentino, G. Pardini, A. T. Coste, J. Bille, and D. Sanglard.** 2006. *CRZ1*, a target of the calcineurin pathway in *Candida albicans*. *Mol. Microbiol.* **59:**1429–1451.
40. **Karnani, N., N. A. Gaur, S. Jha, N. Puri, S. Krishnamurthy, S. K. Goswami, G. Mukhopadhyay, and R. Prasad.** 2004. SRE1 and SRE2 are two specific steroid-responsive modules of *Candida* drug resistance gene 1 (*CDR1*) promoter. *Yeast* **21:**219–239.
41. **Katiyar, S. K., and T. D. Edlind.** 2001. Identification and expression of multidrug resistance-related ABC transporter genes in *Candida krusei*. *Med. Mycol.* **39:**109–116.
42. **Kelly, S. L., A. Arnoldi, and D. E. Kelly.** 1993. Molecular genetic analysis of azole antifungal mode of action. *Biochem. Soc. Trans.* **21:**1034–1038.
43. **Kelly, S. L., D. C. Lamb, D. E. Kelly, J. Loeffler, and H. Einsele.** 1996. Resistance to fluconazole and amphotericin in *Candida albicans* from AIDS patients. *Lancet* **348:**1523–1524.
44. **Kelly, S. L., D. C. Lamb, D. E. Kelly, N. J. Manning, J. Loeffler, H. Hebart, U. Schumacher, and H. Einsele.** 1997. Resistance to fluconazole and cross-resistance to amphotericin B in *Candida albicans* from AIDS patients caused by defective sterol delta5,6-desaturation. *FEBS Lett.* **400:**80–82.
45. **Kofteridis, D. P., R. E. Lewis, and D. P. Kontoyiannis.** 2010. Caspofungin-non-susceptible *Candida* isolates in cancer patients. *J. Antimicrob. Chemother.* **65:**293–295.
46. Reference deleted.
47. **Kontoyiannis, D. P., and R. E. Lewis.** 2002. Antifungal drug resistance of pathogenic fungi. *Lancet* **359:**1135–1144.
48. **Krishnamurthy, S., V. Gupta, R. Prasad, S. L. Panwar, and R. Prasad.** 1998. Expression of *CDR1*, a multidrug resistance gene of *Candida albicans*: transcriptional activation by heat shock, drugs and human steroid hormones. *FEMS Microbiol. Lett.* **160:**191–197.
49. **Kudo, M., M. Ohi, Y. Aoyama, Y. Nitahara, S. K. Chung, and Y. Yoshida.** 2005. Effects of Y132H and F145L substitutions on the activity, azole resistance and spectral properties of *Candida albicans* sterol 14-demethylase P450 (CYP51): a live example showing the selection of altered P450 through interaction with environmental compounds. *J. Biochem.* **137:**625–632.

50. **Lavoie, H., A. Sellam, C. Askew, A. Nantel, and M. Whiteway.** 2008. A toolbox for epitope-tagging and genome-wide location analysis in *Candida albicans*. *BMC Genomics* **9:**578.
51. **Lee, M. K., L. E. Williams, D. W. Warnock, and B. A. Arthington-Skaggs.** 2004. Drug resistance genes and trailing growth in *Candida albicans* isolates. *J. Antimicrob. Chemother.* **53:**217–224.
52. **Liu, T. T., R. E. Lee, K. S. Barker, R. E. Lee, L. Wei, R. Homayouni, and P. D. Rogers.** 2005. Genome-wide expression profiling of the response to azole, polyene, echinocandin, and pyrimidine antifungal agents in *Candida albicans*. *Antimicrob. Agents Chemother.* **49:**2226–2236.
53. **Liu, T. T., S. Znaidi, K. S. Barker, L. Xu, R. Homayouni, S. Saidane, J. Morschhäuser, A. Nantel, M. Raymond, and P. D. Rogers.** 2007. Genome-wide expression and location analyses of the *Candida albicans* Tac1p regulon. *Eukaryot. Cell* **6:**2122–2138.
54. **Lopez-Ribot, J. L., R. K. McAtee, L. N. Lee, W. R. Kirkpatrick, T. C. White, D. Sanglard, and T. F. Patterson.** 1998. Distinct patterns of gene expression associated with development of fluconazole resistance in serial *Candida albicans* isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. *Antimicrob. Agents Chemother.* **42:**2932–2937.
55. **Lyman, C. A., and T. J. Walsh.** 1992. Systemically administered antifungal agents. A review of their clinical pharmacology and therapeutic applications. *Drugs* **44:**9–35.
56. **Lyons, C. N., and T. C. White.** 2000. Transcriptional analyses of antifungal drug resistance in *Candida albicans*. *Antimicrob. Agents Chemother.* **44:**2296–2303.
57. **MacPherson, S., B. Akache, S. Weber, X. De Deken, M. Raymond, and B. Turcotte.** 2005. *Candida albicans* zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. *Antimicrob. Agents Chemother.* **49:**1745–1752.
58. **Martins, M. D., M. Lozano-Chiu, and J. H. Rex.** 1998. Declining rates of oropharyngeal candidiasis and carriage of *Candida albicans* associated with trends toward reduced rates of carriage of fluconazole-resistant *C. albicans* in human immunodeficiency virus-infected patients. *Clin. Infect. Dis.* **27:**1291–1294.
59. **Miyazaki, H., Y. Miyazaki, A. Geber, T. Parkinson, C. Hitchcock, D. J. Falconer, D. J. Ward, K. Marsden, and J. E. Bennett.** 1998. Fluconazole resistance associated with drug efflux and increased transcription of a drug transporter gene, *PDH1*, in *Candida glabrata*. *Antimicrob. Agents Chemother.* **42:**1695–1701.
60. **Miyazaki, T., S. Yamauchi, T. Inamine, Y. Nagayoshi, T. Saijo, K. Izumikawa, M. Seki, H. Kakeya, Y. Yamamoto, K. Yanagihara, Y. Miyazaki, and S. Kohno.** 2010. Roles of calcineurin and Crz1 in antifungal susceptibility and virulence of *Candida glabrata*. *Antimicrob. Agents Chemother.* **54:**1639–1643.
61. **Moran, G., D. Sullivan, J. Morschhäuser, and D. Coleman.** 2002. The *Candida dubliniensis CdCDR1* gene is not essential for fluconazole resistance. *Antimicrob. Agents Chemother.* **46:**2829–2841.
62. **Morschhäuser, J.** 2002. The genetic basis of fluconazole resistance development in *Candida albicans*. *Biochim. Biophys. Acta* **1587:**240–248.
63. **Morschhäuser, J., K. S. Barker, T. T. Liu, J. Blaß-Warmuth, R. Homayouni, and P. D. Rogers.** 2007. The transcription factor Mrr1p controls expression of the *MDR1* efflux pump and mediates multidrug resistance in *Candida albicans*. *PLoS Pathog.* **3:**e164.
64. **Moye-Rowley, W. S.** 2003. Regulation of the transcriptional response to oxidative stress in fungi: similarities and differences. *Eukaryot. Cell* **2:**381–389.
65. **Niimi, M., K. Niimi, Y. Takano, A. R. Holmes, F. J. Fischer, Y. Uehara, and R. D. Cannon.** 2004. Regulated overexpression of *CDR1* in *Candida albicans* confers multidrug resistance. *J. Antimicrob. Chemother.* **54:**999–1006.
66. **Niimi, K., K. Maki, F. Ikeda, A. R. Holmes, E. Lamping, M. Niimi, B. C. Monk, and R. D. Cannon.** 2006. Overexpression of *Candida albicans CDR1*, *CDR2*, or *MDR1* does not produce significant changes in echinocandin susceptibility. *Antimicrob. Agents Chemother.* **50:**1148–1155.
67. **Nolte, F. S., T. Parkinson, D. J. Falconer, S. Dix, J. Williams, C. Gilmore, R. Geller, and J. R. Wingard.** 1997. Isolation and characterization of fluconazole- and amphotericin B-resistant *Candida albicans* from blood of two patients with leukemia. *Antimicrob. Agents Chemother.* **41:**196–199.
68. **Nourani, A., D. Papajova, A. Delahodde, C. Jacq, and J. Subik.** 1997. Clustered amino acid substitutions in the yeast transcription regulator Pdr3p increase pleiotropic drug resistance and identify a new central regulatory domain. *Mol. Gen. Genet.* **256:**397–405.
69. **Oliver, B. G., J. L. Song, J. H. Choiniere, and T. C. White.** 2007. *cis*-Acting elements within the *Candida albicans ERG11* promoter mediate the azole response through transcription factor Upc2p. *Eukaryot. Cell* **6:**2231–2239.
70. **Onyewu, C., F. L. Wormley, Jr., J. R. Perfect, and J. Heitman.** 2004. The calcineurin target, Crz1, functions in azole tolerance but is not required for virulence of *Candida albicans*. *Infect Immun.* **72:**7330–7333.
71. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, and J. D. Sobel.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.
72. **Perea, S., J. L. Lopez-Ribot, W. R. Kirkpatrick, R. K. McAtee, R. A. Santillan, M. Martinez, D. Calabrese, D. Sanglard, and T. F. Patterson.** 2001. Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob. Agents Chemother.* **45:**2676–2684.
73. **Perlin, D. S.** 2009. Antifungal drug resistance: do molecular methods provide a way forward? *Curr. Opin. Infect. Dis.* **22:**568–573.
74. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, D. Ellis, V. Tullio, A. Rodloff, W. Fu, T. A. Ling, and the Global Antifungal Surveillance Group.** 2010. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole as determined by CLSI standardized disk diffusion. *J. Clin. Microbiol.* **48:**1366–1377.
75. **Prasad, R., P. De Wergifosse, A. Goffeau, and E. Balzi.** 1995. Molecular cloning and characterization of a novel gene of *Candida albicans*, *CDR1*, conferring multiple resistance to drugs and antifungals. *Curr. Genet.* **27:**320–329.
76. Reference deleted.
77. **Raymond, M., D. Dignard, A. M. Alarco, K. L. Clark, S. Weber, M. Whiteway, E. Leberer, and D. Y. Thomas.** 2000. Molecular cloning of the *CRM1* gene from *Candida albicans*. *Yeast* **16:**531–538.
78. **Riggle, P. J., and C. A. Kumamoto.** 2006. Transcriptional regulation of *MDR1*, encoding a drug efflux determinant, in fluconazole-resistant *Candida albicans* strains through an Mcm1p binding site. *Eukaryot. Cell* **5:**1957–1968.
79. **Rogers, P. D., and K. S. Barker.** 2003. Genome-wide expression profile analysis reveals coordinately regulated

genes associated with stepwise acquisition of azole resistance in *Candida albicans* clinical isolates. *Antimicrob. Agents Chemother.* **47:**1220–1227.
80. **Rognon, B., Z. Kozovska, A.T. Coste, G. Pardini, and D. Sanglard.** 2006. Identification of promoter elements responsible for the regulation of *MDR1* from *Candida albicans*, a major facilitator transporter involved in azole resistance. *Microbiology* **152:**3701–3722.
81. **Ruhnke, M., A. Eigler, I. Tennagen, B. Geiseler, E. Engelmann, and M. Trautmann.** 1994. Emergence of fluconazole-resistant strains of *Candida albicans* in patients with recurrent oropharyngeal candidosis and human immunodeficiency virus infection. *J. Clin. Microbiol.* **32:** 2092–2098.
82. **Saidane, S., S. Weber, X. De Deken, G. St.-Germain, and M. Raymond.** 2006. *PDR16*-mediated azole resistance in *Candida albicans*. *Mol. Microbiol.* **60:**1546–1562.
83. **Sanglard, D., K. Kuchler, F. Ischer, J. L. Pagani, M. Monod, and J. Bille.** 1995. Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob. Agents Chemother.* **39:**2378–2386.
84. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1996. Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. *Antimicrob. Agents Chemother.* **40:**2300–2305.
85. **Sanglard, D., F. Ischer, M. Monod, and J. Bille.** 1997. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of *CDR2*, a new multidrug ABC transporter gene. *Microbiology* **143:** 405–416.
86. **Sanglard, D., F. Ischer, L. Koymans, and J. Bille.** 1998. Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob. Agents Chemother.* **42:**241–253.
87. **Sanglard, D., F. Ischer, D. Calabrese, P. A. Majcherczyk, and J. Bille.** 1999. The ATP binding cassette transporter gene *CgCDR1* from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. *Antimicrob. Agents Chemother.* **43:**2753–2765.
88. **Sanglard, D., F. Ischer, and J. Bille.** 2001. Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. *Antimicrob. Agents Chemother.* **45:**1174–1183.
89. **Sanglard, D.** 2002. Resistance of human fungal pathogens to antifungal drugs. *Curr. Opin. Microbiol.* **5:**379–385.
90. **Sanglard, D., F. Ischer, O. Marchetti, J. Entenza, and J. Bille.** 2003. Calcineurin A of *Candida albicans*: involvement in antifungal tolerance, cell morphogenesis and virulence. *Mol. Microbiol.* **48:**959–976.
91. **Sanglard, D., A. Coste, and S. Ferrari.** 2009. Antifungal drug resistance mechanisms in fungal pathogens from the perspective of transcriptional gene regulation. *FEMS Yeast Res.* **9:**1029–1050.
92. **Sanguinetti, M., B. Posteraro, B. Fiori, S. Ranno, R. Torelli, and G. Fadda.** 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. *Antimicrob. Agents Chemother.* **49:**668–679.
93. **Schubert, S., P. D. Rogers, and J. Morschhäuser.** 2008. Gain-of-function mutations in the transcription factor *MRR1* are responsible for overexpression of the *MDR1* efflux pump in fluconazole-resistant *Candida dubliniensis* strains. *Antimicrob. Agents Chemother.* **52:**4274–4280.
94. **Sellam, A., F. Tebbji, and A. Nantel.** 2009. Role of Ndt80p in sterol metabolism regulation and azole resistance in *Candida albicans*. *Eukaryot. Cell* **8:**1174–1183.
95. **Sellam, A., C. Askew, E. Epp, H. Lavoie, M. Whiteway, and A. Nantel.** 2009. Genome-wide mapping of the coactivator Ada2p yields insight into the functional roles of SAGA/ADA complex in *Candida albicans*. *Mol. Biol. Cell* **20:**2389–2400.
96. **Selmecki, A., A. Forche, and J. Berman.** 2006. Aneuploidy and isochromosome formation in drug-resistant *Candida albicans*. *Science* **313:**367–370.
97. Reference deleted.
98. **Shukla, S., P. Saini, Smriti, S. Jha, S. V. Ambudkar, and R. Prasad.** 2003. Functional characterization of *Candida albicans* ABC transporter Cdr1. *Eukaryot. Cell* **2:** 1361–1375.
99. **Shukla, S., V. Rai, D. Banerjee, and R. Prasad.** 2006. Characterization of Cdr1p, a major multidrug efflux protein of *Candida albicans*: purified protein is amenable to intrinsic fluorescence analysis. *Biochemistry* **45:**2425–2435.
100. **Silver, P. M., B. G. Oliver, and T. C. White.** 2004. Role of *Candida albicans* transcription factor Upc2p in drug resistance and sterol metabolism. *Eukaryot. Cell* **3:**1391–1397.
101. **Slain, D.** 1999. Lipid-based amphotericin B for the treatment of fungal infections. *Pharmacotherapy* **19:**306–323.
102. **Smith, W. L., and T. D. Edlind.** 2002. Histone deacetylase inhibitors enhance *Candida albicans* sensitivity to azoles and related antifungals: correlation with reduction in *CDR* and *ERG* upregulation. *Antimicrob. Agents Chemother.* **46:**3532–3539.
103. **Sucher, A. J., E. B. Chahine, H. E. Balcer.** 2009. Echinocandins: the newest class of antifungals. *Ann. Pharmacother.* **43:**1647–1657.
104. **Talibi, D., and M. Raymond.** 1999. Isolation of a putative *Candida albicans* transcriptional regulator involved in pleiotropic drug resistance by functional complementation of a *pdr1 pdr3* mutation in *Saccharomyces cerevisiae*. *J. Bacteriol.* **181:**231–240.
105. **Thakur, J. K., H. Arthanari, F. Yang, S. J. Pan, X. Fan, J. Breger, D. P. Frueh, K. Gulshan, D. K. Li, E. Mylonakis, K. Struhl, W. S. Moye-Rowley, B. P. Cormack, G. Wagner, and A. M. Näär.** 2008. A nuclear receptor-like pathway regulating multidrug resistance in fungi. *Nature* **452:**604–609.
106. **Torelli, R., B. Posteraro, S. Ferrari, M. La Sorda, G. Fadda, D. Sanglard, and M. Sanguinetti.** 2008. The ATP-binding cassette transporter-encoding gene *CgSNQ2* is contributing to the *CgPDR1*-dependent azole resistance of *Candida glabrata*. *Mol. Microbiol.* **68:**186–201.
107. **Traeder, C., S. Kowoll, and K. Arastéh.** 2008. *Candida* infection in HIV positive patients 1985–2007. *Mycoses* **51**(Suppl. 2)**:**58–61.
108. **Tsai, H. F., A. A. Krol, K. E. Sarti, and J. E. Bennett.** 2006. *Candida glabrata PDR1*, a transcriptional regulator of a pleiotropic drug resistance network, mediates azole resistance in clinical isolates and petite mutants. *Antimicrob. Agents Chemother.* **50:**1384–1392.
109. **Tsao, S., F. Rahkhoodaee, and M. Raymond.** 2009. Relative contributions of the *Candida albicans* ABC transporters Cdr1p and Cdr2p to clinical azole resistance. *Antimicrob. Agents Chemother.* **53:**1344–1352.
110. **Vermitsky, J. P., and T. D. Edlind.** 2004. Azole resistance in *Candida glabrata*: coordinate upregulation of multidrug transporters and evidence for a Pdr1-like transcription factor. *Antimicrob. Agents Chemother.* **48:** 3773–3781.
111. **Vermitsky, J. P., K. D. Earhart, W. L. Smith, R. Homayouni, T. D. Edlind, and P. D. Rogers.** 2006. Pdr1 regulates multidrug resistance in *Candida glabrata*: gene disruption and genome-wide expression studies. *Mol. Microbiol.* **61:**704–722.

112. **Vik, A., and J. Rine.** 2001. Upc2p and Ecm22p, dual regulators of sterol biosynthesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **21:**6395–6405.
113. **Wada, S., M. Niimi, K. Niimi, A. R. Holmes, B. C. Monk, R. D. Cannon, and Y. Uehara.** 2002. *Candida glabrata* ATP-binding cassette transporters Cdr1p and Pdh1p expressed in a *Saccharomyces cerevisiae* strain deficient in membrane transporters show phosphorylation-dependent pumping properties. *J. Biol. Chem.* **277:** 46809–46821.
114. **Wang, J. S., Y. L. Yang, C. J. Wu, K. J. Ouyang, K. Y. Tseng, C. G. Chen, H. Wang, and H. J. Lo.** 2006. The DNA-binding domain of CaNdt80p is required to activate *CDR1* involved in drug resistance in *Candida albicans*. *J. Med. Microbiol.* **55:**1403–1411.
115. **White, T. C.** 1997. The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14α demethylase in *Candida albicans*. *Antimicrob. Agents Chemother.* **41:**1488–1494.
116. **White, T. C., K. A. Marr, and R. A. Bowden.** 1998. Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. *Clin. Microbiol. Rev.* **11:**382–402.
117. **Wirsching, S., S. Michel, G. Kohler, and J. Morschhäuser.** 2000. Activation of the multiple drug resistance gene *MDR1* in fluconazole-resistant, clinical *Candida albicans* strains is caused by mutations in a *trans*-regulatory factor. *J. Bacteriol.* **182:**400–404.
118. **Wirsching, S., G. P. Moran, D. J. Sullivan, D. C. Coleman, and J. Morschhäuser.** 2001. *MDR1*-mediated drug resistance in *Candida dubliniensis*. *Antimicrob. Agents Chemother.* **45:**3416–3421.
119. **Yang, X., D. Talibi, S. Weber, G. Poisson, and M. Raymond.** 2001. Functional isolation of the *Candida albicans FCR3* gene encoding a bZip transcription factor homologous to *Saccharomyces cerevisiae* Yap3p. *Yeast* **18:** 1217–1225.
120. **Zhang, X., M. De Micheli, S. T. Coleman, D. Sanglard, and W. S. Moye-Rowley.** 2000. Analysis of the oxidative stress regulation of the *Candida albicans* transcription factor, Cap1p. *Mol. Microbiol.* **36:**618–629.
121. **Znaidi, S., X. De Deken, S. Weber, T. Rigby, A. Nantel, and M. Raymond.** 2007. The zinc cluster transcription factor Tac1p regulates *PDR16* expression in *Candida albicans*. *Mol. Microbiol.* **66:**440–452.
122. **Znaidi, S., K. S. Barker, S. Weber, A. M. Alarco, T. T. Liu, G. Boucher, P. D. Rogers, and M. Raymond.** 2009. Identification of the *Candida albicans* Cap1p regulon. *Eukaryot. Cell* **8**:806–820.
123. **Znaidi, S., S. Weber, O. Z. Al-Abdin, P. Bomme, S. Saidane, S. Drouin, S. Lemieux, X. De Deken, F. Robert, and M. Raymond.** 2008. Genomewide location analysis of *Candida albicans* Upc2p, a regulator of sterol metabolism and azole drug resistance. *Eukaryot. Cell* **7:**836–847.

CANDIDIASIS, EVOLVING DIAGNOSTICS, AND TREATMENT PARADIGMS

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch26

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Mucosal Candidiasis

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OVERVIEW

Mucosal candidiasis is extremely common and vastly more common than invasive, systemic candidiasis, although the latter receives far more attention due to the accompanying mortality. Nevertheless, mucosal candidiasis is responsible for considerable morbidity in hospitalized, often immunocompromised hosts but also in ambulatory, otherwise entirely healthy subjects. Although considerable progress has been made in the understanding of pathogenesis of mucosal candidiasis, considerable deficits in our knowledge persist, accompanied by dynamic changes in microbiology of the responsible *Candida* species and antifungal drug susceptibility, including drug resistance. Regrettably, new anti-*Candida* drugs for mucosal candidiasis are in short supply, and refractory, especially recurrent, mucosal candidiasis is common, creating an enormous therapeutic challenge. The failure of antifungal agents for refractory mucosal candidiasis provides an opportunity for nondrug therapeutic alternative approaches, including vaccine development.

INTRODUCTION

Mucosal candidiasis involving the oral, gastrointestinal, and vaginal mucosae represents the commonest forms of superficial candidiasis and contrasts significantly with systemic and visceral candidiasis and disease. Although involving widely different anatomical sites with profoundly divergent physiological influences, the forms of mucosal candidiasis at the various sites have much in common with regard to pathophysiology, diagnostic principles, clinical manifestations, and therapy. Much information can be extrapolated from one anatomical site to another, but there are limits to their similarities. Mucosal candidiasis is so called because it entails both physiological colonization, usually asymptomatic, and symptomatic disease states. Common to all sites of mucosal candidiasis is the rarity of local *Candida* tissue invasion, candidemia, and disseminated candidiasis. Host defense mechanisms directed against invasive candidiasis differ profoundly from those protecting mucosal sites, although most defects may coexist and patients may present with concomitant superficial and deep candidiasis, synonymous with mucosal and invasive candidiasis. The purpose of this chapter is to review clinical aspects of mucosal candidiasis, including diagnosis and therapy, without emphasizing immunopathogenesis, which is reviewed elsewhere (see chapter 11).

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ORAL CANDIDIASIS

Epidemiology

Oral candidiasis (OC) is a commonly encountered condition in clinical practice, almost invariably associated with underlying disease or risk factors. It has been recognized since ancient times; it was noted by Hippocrates in patients with severe debilitation (30). However, it was not until 1846 that Fredrik Berg recognized *Candida* as the etiologic agent (30).

Many diseases have been associated with OC, including human immunodeficiency virus (HIV) infection, hematologic malignancies, poorly controlled diabetes, xerostomia, and various immunodeficiencies, especially chronic mucocutaneous candidiasis (CMC) (25, 30). Multiple syndromes have been described in CMC, with acquired and inherited forms (25). In addition, risk factors include steroid use (oral and inhaled), antibiotics, head and neck radiation therapy, chemotherapy-induced neutropenia, and dentures (20, 30, 54). Incidence of infection varies with underlying disease; up to 90% of HIV-infected patients develop the disease, while it is relatively uncommon in diabetics without other risk factors (56). In addition, drug-resistant infection is seen commonly in HIV patients, particularly those with advanced immunosuppression and prior azole exposure. A study of over 1,400 HIV-positive patients by Nacher et al. showed that the incidence of OC may be increased in the first 2 months after starting highly active antiretroviral therapy, suggesting a possible role in immune reconstitution syndrome, though specific patient experiences were not described (35). In the elderly, OC is associated with xerostomia, dentures, and use of steroids (18).

Candida albicans is the most common species isolated from oral lesions, with *C. glabrata* and *C. tropicalis* less frequently seen (56). Other species, including *C. parapsilosis*, *C. krusei*, *C. dubliniensis*, and *C. guilliermondii*, are rare.

Pathogenesis

The pathogenic mechanisms that are responsible for development of OC are incompletely understood, though they are likely multifactorial. Both host and pathogen factors have been identified. T cells, specifically $CD4^+$, appear to regulate phagocyte responses, which are important in controlling yeast in oral mucosa (60), hence the predisposition to OC in AIDS patients. Other host factors have also been implicated, including Toll-like receptors 2 and 4 (TLR2 and -4) and alteration of immunoglobulin A (IgA) levels (6, 14, 60). Multiple potential virulence factors in *Candida* have been elucidated that likely contribute to pathogenesis in mucosal disease. These include production of biofilms, mucosal adhesion, hyphal growth, and secreted aspartyl proteases (22, 36, 38). C. *albicans* has been shown to have increased adherence to mucosa over that of non-C. *albicans Candida*, suggesting increased pathogenicity (22).

Clinical Manifestations

OC is often asymptomatic or mildly symptomatic, with patients complaining of burning pain and odynophagia. Occasionally these symptoms may be severe enough to cause significant reduction in oral intake and weight loss. A variety of clinical presentations may be seen. The most common is pseudomembranous candidiasis, which may be acute or chronic (56). This is characterized by whitish, curd-like plaques on mucosal surfaces that can be extensive and may be removed, revealing erythematous mucosa underneath. The extent of disease may not correlate with symptoms.

Another frequently seen presentation is that of chronic atrophic stomatitis, or denture stomatitis (56). Erythematous lesions are observed with burning pain in the denture area. Angular cheilitis, or perleche, may be associated with *Candida* as well. Less frequent manifestations include chronic hyperplastic candidiasis, with lesions on the cheek, particularly in men who smoke, and midline glossitis, characterized by erythema and loss of papillae in the center of the tongue (56).

Diagnosis

In a typical host, the diagnosis of OC is often presumptive based on clinical features. However, other conditions may be difficult to differentiate, such as mucositis, hairy leukoplakia, and lymphoma (39). While cultures may be obtained by swab to confirm suspicion, these are not sufficient, since *Candida* may be a commensal organism. Examining scrapings of lesions with 10% KOH preparation to visualize yeasts and pseudohyphae is the most reliable method of diagnosis (56).

Treatment

Several therapies are available for the treatment of OC that are generally well tolerated and effective (Table 1). Duration of therapy is usually 7 to 14 days. Topical preparations of azoles with clotrimazole or miconazole are effective but need to be taken two to five times in a day. Recently, a miconazole mucoadhesive buccal tablet has been developed that provides once-daily dosing (4). The advantages to these topical agents are the absence of significant adverse effects and drug interactions. Topical polyenes such as nystatin and amphotericin B are often not well tolerated and must be administered several times a day (56), though they may be useful in cases of resistant infection (13). Topical chlorhexidine has been effective in cases of denture stomatitis due to *Candida* species (34).

Systemic therapy with oral azoles is highly effective and well tolerated, especially fluconazole, which remains the drug of choice. It is taken once a day and has excellent oral bioavailability and relatively few drug interactions. However, resistance is frequently encountered, particularly in patients with advanced HIV infection and prolonged exposure to the drug. Alternatives in patients not responding to fluconazole due to clinical resistance include itraconazole, posaconazole, and voriconazole, all of which have been shown to be effective (26, 28). Posaconazole in particular has been shown to be useful in cases of fluconazole-refractory thrush, up to 1 year (47, 57). In patients with recurrent disease, chronic fluconazole is effective in preventing disease and may not be associated with increased rates of resistance (17).

Occasionally patients may require intravenous therapy with echinocandins or amphotericin B. Echinocandins are preferred where available due to ease of administration (once daily), low incidence of side effects, and negligible drug interactions. Multiple studies with HIV-positive patients have confirmed their effectiveness, especially in azole-refractory disease (3, 8, 24, 27).

ESOPHAGEAL CANDIDIASIS

Esophageal candidiasis (EC) is seen less commonly than OC, though usually in more immunocompromised patients (56, 61). It may occur in the absence of OC, and in some studies this was the most common presentation (56). The HIV epidemic dramatically increased the incidence of EC, and most clinical trials have been conducted with HIV-positive patients. Although it may be asymptomatic, common complaints include dysphagia, odynophagia, and retrosternal chest pain. Diagnosis is made by visualization on esophagogastroduodenoscopy of typical white to cream-colored plaques that may progress to ulceration in severe cases. Brushings and biopsy samples are taken to confirm the diagnosis, since culture alone could reflect colonization only. A normal esophagogastroduodenoscopy exam essentially excludes the diagnosis (62).

Systemic therapy is required, as topical agents are rarely effective. If tolerated, oral therapy may be possible and effective; however, most require initial intravenous therapy. Fluconazole is the drug of choice as with OC, though longer treatment courses may be required. Alternatives include itraconazole, posaconazole, and voriconazole, particularly for refractory disease (2, 65). Echinocandins and amphotericin B may be required for cases associated with highly resistant strains of *Candida* (9, 58, 59).

VULVOVAGINAL CANDIDIASIS

Epidemiology

Worldwide, *Candida* vaginitis is the second most common vaginal infection. During the childbearing years, 75% of women experience at least one episode of vulvovaginal candidiasis (VVC), and 40 to 50% of these women experience a second attack (23). *Candida* is isolated from the genital tract of approximately 10 to 20% of asymptomatic, healthy women of childbearing age. Approximately 8% of women experience multiple episodes of VVC defined as recurrent VVC (RVVC) (four or more episodes per year).

Pathogenesis and Microbiology

Candida organisms gain access to the vagina from the adjacent perianal area and then adhere to vaginal epithelial

TABLE 1 Recommendations for prevention and treatment of mucosal candidiasis[a]

Treatment for mucosal candidiasis
- OC: initial episodes (7- to 14-day treatment)
  - Preferred therapy
    - Fluconazole, 100 mg p.o. daily (AI) or
    - Clotrimazole troches, 10 mg p.o. 5 times daily (BII) or
    - Nystatin suspension, 4–6 ml q.i.d. or 1 or 2 flavored pastilles 4 or 5 times daily (BII)
    - Miconazole mucoadhesive tablet, p.o. daily (BII)
  - Alternative therapy
    - Itraconazole oral solution, 200 mg p.o. daily (BI) or
    - Posaconazole oral solution, 400 mg p.o. b.i.d. once and then 400 mg daily (BI)

- EC (duration of therapy, 14–21 days)
  - Preferred therapy
    - Fluconazole, 100 mg (up to 400 mg) p.o. or i.v. daily (AI)
    - Itraconazole oral solution, 200 mg p.o. daily (AI)
  - Alternative therapy
    - Voriconazole, 200 mg p.o. or i.v. b.i.d. (BI)
    - Posaconazole, 400 mg p.o. b.i.d. (BI)
    - Caspofungin, 50 mg i.v. daily (BII)
    - Micafungin, 150 mg i.v. daily (BII)
    - Anidulafungin, 100 mg i.v. once and then 50 mg i.v. daily (BII)
    - Amphotericin B deoxycholate, 0.6 mg/kg i.v. daily (BII)

- Fluconazole-refractory OC or EC
  - Preferred therapy
    - Itraconazole oral solution, ≥200 mg p.o. daily (AII)
    - Posaconazole oral solution, 400 mg p.o. b.i.d. (AII)
  - Alternative therapy
    - Amphotericin B deoxycholate, 0.3 mg/kg i.v. daily (BII)
    - Lipid formulation of amphotericin B, 3–5 mg/kg i.v. daily (BII)
    - Anidulafungin, 100 mg i.v. once and then 50 mg i.v. daily (BII)
    - Caspofungin, 50 mg i.v. daily (BII)
    - Micafungin, 150 mg i.v. daily (BII)
    - Voriconazole, 200 mg p.o. or i.v. b.i.d. (BII)
- Fluconazole-refractory oropharyngeal candidiasis (not esophageal)[b]
    - Amphotericin B oral suspension, 100 mg/ml (not available in the United States)–1 ml p.o. q.i.d. (BII)

- Uncomplicated VVC
  - Preferred therapy
    - Oral fluconazole, 150 mg for 1 dose (AI)
    - Topical azoles (clotrimazole, butoconazole, miconazole, tioconazole, or terconazole) for 3–7 days (AI)
  - Alternative therapy
    - Itraconazole oral solution, 200 mg p.o. daily for 3–7 days (BII)

Chronic suppressive therapy
- Suppressive therapy is usually not recommended (AI) unless patients have frequent or severe recurrences. Antiretroviral therapy is recommended (AI).
- If used, it is reason to discontinue therapy if CD4 > 200 cells/μl.
- If decision is to use suppressive therapy:
  - OC
    - Fluconazole, 100 mg p.o. t.i.w. (AI)
    - Itraconazole oral solution, 200 mg p.o. daily (CI)
  - EC
    - Fluconazole, 100–200 mg p.o. t.i.w. (AI)
    - Posaconazole, 400 mg p.o. b.i.d. (BII)
  - VVC
    - Fluconazole, 150 mg p.o. once weekly (AI)
    - Daily topical azole (CII)

Other considerations
- Chronic or prolonged use of azoles might promote development of resistance
- Higher relapse rate of EC with echinocandins than with fluconazole has been reported

[a]Abbreviations: p.o., per os; q.i.d., four times a day; b.i.d., twice a day; i.v., intravenously; t.i.w., three times a week. AI, BII, etc., are scores for strength of evidence for ranking recommendations per the Infectious Diseases Society of America (42a).

[b]Patients with fluconazole-refractory oropharyngeal candidiasis or EC who responded to echinocandin should be started on voriconazole or posaconazole for secondary prophylaxis until antiretroviral therapy produces immune reconstitution (CI).

cells. *Candida albicans* adheres to vaginal epithelial cells in significantly greater numbers than non-*C. albicans Candida* species (48).

Several factors, including pregnancy, oral contraceptives with a high estrogen content, and uncontrolled diabetes mellitus, are associated with increased rates of asymptomatic vaginal colonization with *Candida* as well as *Candida* vaginitis (Fig. 1). The hormonal dependence of VVC is illustrated by the facts that *Candida* is seldom isolated from premenarchial girls and the prevalence of *Candida* vaginitis is lower after menopause, except in women taking hormone replacement therapy. Other predisposing factors include corticosteroids, antimicrobial therapy, intrauterine devices, and high frequency of coitus (48).

Candida organisms exist as benign but adapted colonizers and transform to invasive mode with expression of many additional genes (44). The innate immune system recognizes pathogen-associated molecular patterns through pattern recognition receptors, which transduces downstream signaling to activate host defenses. In addition, *C. albicans* releases soluble factors to actively potentiate interleukin 6 (IL-6) and IL-8 production by mononuclear cells (7). Germination of *Candida* enhances colonization and tissue invasion. Factors that enhance or facilitate germination, such as estrogen therapy and pregnancy, tend to precipitate symptomatic vaginitis, whereas epithelial cell innate immunity that inhibits germination prevents acute vaginitis in women who are asymptomatic carriers of *Candida*. A clinical precipitating factor that explains the transformation from asymptomatic carriage to symptomatic vaginitis is identified in only a few patients (Fig. 1).

During pregnancy, the incidence of clinical episodes often reaches a maximum in the third trimester, but symptomatic recurrences are common throughout pregnancy. The high levels of reproductive hormones are thought to increase the glycogen content of the vaginal environment and provide a carbon source for *Candida* growth and germination. Estrogens enhance vaginal epithelial cell avidity for *Candida* adherence, and a yeast cytosol receptor or binding system for female reproductive hormones has been documented. In addition, estrogens enhance yeast-mycelial transformation. Low-estrogen oral contraceptives may also cause an increase in *Candida* vaginitis. Hormone replacement therapy may contribute to vaginitis in postmenopausal women. Vaginal colonization with *Candida* is more common in diabetic patients, and poorly controlled diabetes predisposes to symptomatic vaginitis. Glucose tolerance tests have been recommended for women with RVVC; however, the yield is low and testing is not justified in otherwise healthy premenopausal women. Diets high in refined sugar may precipitate symptomatic *Candida* vaginitis (48).

Symptomatic VVC is often observed during or after use of systemic or intravaginal antibiotics (43). Although no antimicrobial agent is free of this complication, the broad-spectrum antibiotics are mainly responsible. They may act by eliminating the proposed but "unproven" protective vaginal bacterial flora. *Lactobacillus* species normally found in the vagina have been thought to provide a natural defensive mechanism suppressing the proliferation and pathogenic potential of *Candida*; however, no evidence supports this concept, and many studies reached the opposite conclusion, namely, that vaginal H_2O_2-producing *Lactobacillus* colonization is associated with increased risk of symptomatic VVC (32). Most women taking antibiotics do not develop *Candida* vaginitis, and vaginal depletion of *Lactobacillus* spp. is not associated with increased risk of VVC. Environmental factors that predispose to *Candida* vaginitis may include tight, poorly ventilated clothing and nylon underclothing,

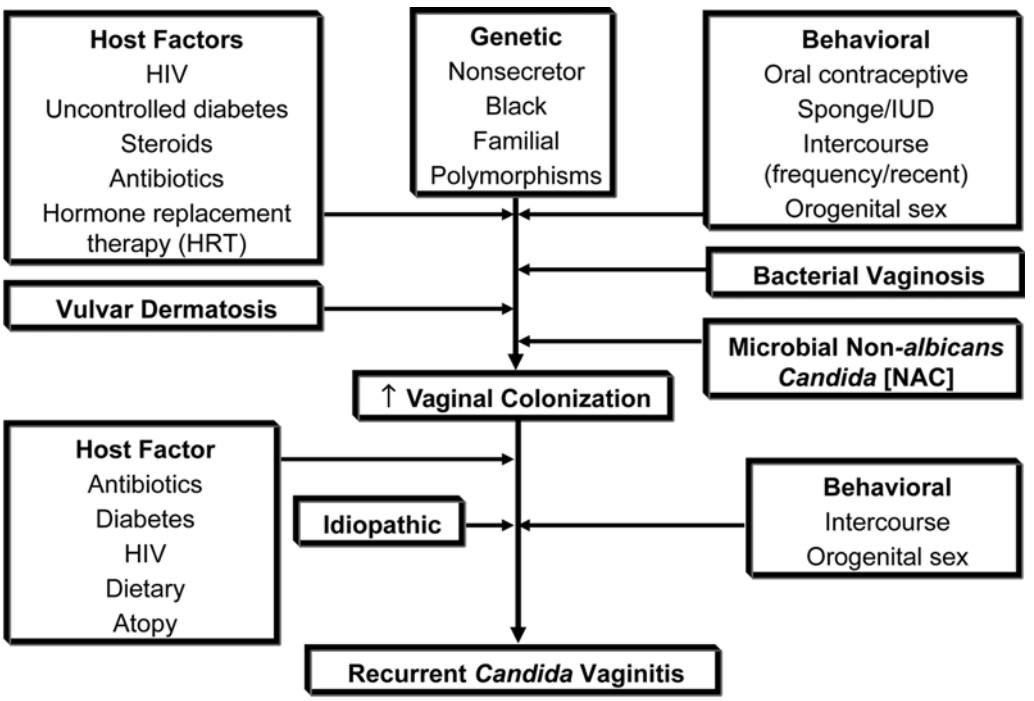

FIGURE 1 Pathogenesis of RVVC. IUD, intrauterine device. 10.1128/9781555817176.ch26f1

which increase perineal moisture and temperature. Chemical contact, local allergy, and hypersensitivity reactions may also predispose to symptomatic vaginitis. Iron deficiency is said to predispose to *Candida* infection, but there is no evidence to support this view (48).

In patients who are debilitated or immunosuppressed, OC and vaginal candidiasis correlate well with reduced cell-mediated immunity, as evident in CMC and AIDS. Based on these observations, T lymphocytes add to normal, effective innate vaginal defense mechanisms preventing mucosal invasion by *Candida*. The role of the vaginal and systemic immune system as a protective best defense mechanism, i.e., anti-*Candida* immunity, as well as its role in the pathogenesis of vaginitis, especially recurrent disease, is beyond the scope of this review. Several recent reviews are available (1, 5).

Several theories have been proposed to explain RVVC (1, 48). The intestinal-reservoir theory is based on recovery of *Candida* on rectal culture for almost 100% of women with VVC. DNA typing of vaginal and rectal cultures obtained simultaneously usually reveals identical strains. However, other studies have shown a lower concordance between rectal and vaginal cultures for patients with RVVC. In a maintenance study of women with RVVC who were receiving ketoconazole, *Candida* vaginitis often recurred with no evidence of *Candida* in the rectum. Also, oral nystatin, which reduces intestinal yeast carriage, fails to prevent RVVC. Repeated reintroduction of yeast into the vagina from the gut is therefore no longer considered responsible for recurrent *Candida* vaginitis.

The sexual-transmission theory is based on the occurrence of penile colonization with *Candida* organisms, which are present in approximately 20% of male partners of women with RVVC, and the observation that infected partners often carry identical *Candida* strains. Oral colonization of partners with an identical strain of *Candida* also occurs and may be a source of orogenital transmission. However, in most studies involving treatment of partners, there was no reduction in the frequency of episodes of vaginitis.

The vaginal-relapse theory to explain RVVC is currently favored, in which antifungal therapy reduces the number of *Candida* organisms and alleviates signs and symptoms of inflammation; however, the eradication of *Candida* from the vagina is incomplete because most agents are fungistatic. Small numbers of organisms persist in the vagina, resulting in continued carriage of the organism, and when local host conditions permit, the colonizing organisms increase in number and undergo mycelial transformation and together with the eventual host response result in a new clinical episode of symptomatic vaginitis. Drug resistance is seldom responsible for RVVC in women infected with *C. albicans*. Current vaginal-relapse theories regarding the pathogenesis of RVVC include qualitative and quantitative deficiency in the normal protective vaginal bacterial flora (unproven) and an acquired, but genetically determined, antigen-specific deficiency in or excessive aberrant local T-lymphocyte function that permits unchecked yeast proliferation and germination. Reduced T-lymphocyte reactivity to *Candida* antigen may result from the elaboration of prostaglandin E_2 by the patient's macrophages, which blocks *Candida* antigen-induced lymphocyte proliferation, possibly by inhibiting IL-2 production. Abnormal macrophage function could be the result of histamine produced as a consequence of local IgE *Candida* antibodies or a serum factor (64). Recent evidence of genetic factors that influence epithelial cell susceptibility to colonization and activation of the innate immune system (dectin-1 and mannose-binding lectin) are likely critical to pathogenesis of RVVC (10, 12, 16, 19). The proposed role of mannose-binding lectin is, however, controversial (33). The interaction of *C. albicans* and the innate host defense system of the vagina has been the main focus of investigation in the last decade. Fidel has emphasized the elaboration of antiadherence molecules by normal estrogen-stimulated vaginal epithelial cells, which serve to control vaginal yeast colonization and proliferation (14). Recognition of *C. albicans* by the innate host defense system is mediated by pattern recognition from TLR and lectin like-receptor families (37). Mannans from the *Candida* cell wall are recognized by the mannose receptor, and TLR4 and TLR2 recognize phospholipomannan and collaborate with the β-glucan receptor dectin-1 in the stimulation of cytokine production (15). Dectin-1 amplifies TLR2- and TLR4-induced cytokine production by human cells, including tumor necrosis factor and, in addition, IL-17, IL-6, and IL-10 (19). A relationship between human dectin-1 deficiency and mucocutaneous fungal infection is now established (12).

The role of *C. albicans*-induced biofilm in vivo in pathogenesis of *Candida* colonization, in vaginitis (especially RVVC), and possibly as a factor in determining azole resistance is currently under investigation (21). Elevated vaginal concentrations of hyaluronan, which promotes proinflammatory immune system activation and function, have recently been reported in association with RVVC (29).

Clinical Manifestations

Vulvar pruritus is the most common symptom of VVC. Vaginal discharge is often minimal and sometimes absent, and although described as being typically "cottage cheese-like" in character, the discharge may vary from watery to homogeneously thick. Vaginal soreness, irritation, vulvar burning, dyspareunia, and external dysuria are other common symptoms. If there is an odor, it is minimal and not offensive. Characteristically, the symptoms are exacerbated during the week before the onset of menses. The onset of menstrual flow brings some relief. Examination reveals erythema and swelling of the labia and vulva, often with discrete pustulopapular peripheral lesions. The cervix is normal and the vaginal mucosa is erythematous, with adherent whitish discharge. VVC, specifically *Candida* vaginitis, is associated with increased vaginal HIV RNA levels (46).

Diagnosis

For most symptomatic patients with VVC, diagnosis is readily made on the basis of microscopic examination of vaginal secretions. A wet mount with saline has a sensitivity, however, of only 40 to 60%. A preparation of 10% potassium hydroxide (KOH) is more sensitive (50 to 70%) in identifying the presence of yeast cells and hyphae. Patients with *Candida* vaginitis have a normal vaginal pH, 4.0 to 4.5. A pH of >4.5 suggests the possibility of bacterial vaginosis, trichomoniasis, or mixed infection. Routine cultures for microscopy-positive patients are unnecessary, but vaginal culture should be performed in pH-normal, suspicious cases with negative microscopy. A positive culture does not necessarily indicate that *Candida* is responsible for the vaginal symptoms. The culture medium routinely used includes Sabouraud's dextrose agent Nickerson; however, the ready-to-use chromogenic media allow culture and identification and have increasingly been used (42).

There are no reliable commercially available serological techniques for the diagnosis of symptomatic VVC. Earlier commercial tests, including latex agglutination slide tests, were less sensitive than culture; however, newer methods

recently reported indicate high sensitivity and specificity in preliminary studies, hold great promise, but have yet to be validated in robust clinical studies (31). Although PCR-based diagnosis is now increasingly used, its utility is unknown except in excluding the diagnosis of VVC. PCR testing is more expensive and although more sensitive than culture has not shown clinical advantage (55).

Treatment of VVC

Treatment of VVC predominantly involves use of the imidazole and triazole agents available as topical or oral formulations (Table 1). Azoles achieve higher success rates, even over shorter durations of therapy, than nystatin vaginal suppositories or creams. No evidence exists that the choice of formulation of the topical azoles influences cure rates (40). Topical agents previously prescribed for 7 to 14 days are now available as single-dose or short-course (3- to 5-day) regimens (48). Topical azoles, when appropriately prescribed, are remarkably free of systemic side effects and toxicity, especially in pregnancy (66).

The oral azoles used for systemic therapy are ketoconazole, itraconazole, and fluconazole. Oral azoles have been shown to be at least as effective as topical agents, are more convenient and more popular among users, but, although free of local side effects, introduce the possibility of systemic adverse effects, especially ketoconazole (48, 49). In selecting an antifungal agent, it is useful to define VVC as uncomplicated or complicated disease (50). The majority of episodes of VVC are uncomplicated (90%). These are sporadic, mild to moderate infections caused by *C. albicans* that occur in healthy hosts who lack predisposing factors. Uncomplicated infections can be successfully treated with any of the available topical or oral antifungal agents, including short-course and single-dose regimens.

Complicated infections are defined as those that (i) have a moderate to severe clinical presentation, (ii) are recurrent in nature (≥4 episodes per year), (iii) are caused by non-*C. albicans Candida* species, or (iv) occur in hosts with predisposing factors, e.g., diabetic patients with poor glucose control. Complicated infections are far less likely to respond to abbreviated courses of therapy (50) and should be treated more intensively for 7 to 14 days in order to achieve a clinical response. In a study of almost 500 women with complicated VVC, prolonging fluconazole therapy by adding a second dose of 150 mg 72 h after the initial dose resulted in significantly higher clinical and mycological cure rates in women with severe VVC (51). Non-*C. albicans Candida* species, especially *C. glabrata*, are less susceptible in vitro to azoles, and VVC caused by these species is less likely to respond clinically, especially to short-course azole therapy. Encouraging results have been obtained with boric acid (600-mg capsules) given vaginally daily for 14 days or topical 17% flucytosine (52). Most patients with VVC caused by *C. glabrata* do not require therapy: the yeast colonizing the lower genital tract serves as an innocent bystander, and another cause for symptoms can be found.

VVC in HIV-infected women is incompletely understood. One large study found it to behave in a fashion similar to that in seronegative women (45). Vaginal carriage of *Candida* is more common in HIV-seropositive women, but symptomatic VVC was not more frequent or was modestly increased only and did not increase with progressive immunosuppression. Others have noted increased rates of VVC with increasing immunosuppression (11). The differences in results may be due to differences in study design and diagnostic criteria (45, 63). Longitudinal cohort studies of vaginal candidiasis in HIV-positive women did show a progressive increase in colonization with *C. glabrata* and diminished fluconazole susceptibility (41). Therapy of VVC in HIV-infected women remains the same as that for seronegative women.

RVVC in HIV-negative women is usually caused by susceptible strains of *C. albicans*, and resistance is rarely encountered. Although more intensive prolonged induction therapy lasting up to 14 days invariably induces remission, the fungistatic nature of the available agents combined with persistence of the underlying host predisposing process makes relapse within 3 months almost inevitable unless a maintenance prophylactic antifungal regimen is employed. The regimen used most often is fluconazole at 150 mg weekly, achieving clinical remission in excess of 90% of women; however, following cessation of suppressive prophylaxis, there is a 50% relapse, reverting to the same patterns of RVVC episodes (53). Clinicians invariably restart fluconazole induction and prophylactic maintenance therapy.

FUTURE CHALLENGES

New Diagnostics

Although *Candida* species are readily identifiable by both microscopy and conventional culture technique, many clinicians no longer possess adequate expertise in clinical diagnosis using traditional methodologies. Microscopes are no longer widely available, and expertise has atrophied over the last few years. While culture reliably indicates the presence of organisms at the mucosal site and quantitative cultures can be obtained, cultures invariably require a 72-h period for a reliable answer. Clinicians need a point-of-care test for rapid, reliable diagnosis. The Affirm test using a DNA probe provides a reliable answer in approximately 2 to 4 h; however, it is not inexpensive, has less-than-optimal sensitivity, and does not identify to the species level (48). A paucity of rapid diagnostic tests based upon antigen detection using immunologic reactions have been studied, and few tests have reached commercial development; finally, currently available technologies do not provide sensitivity in excess of 80 to 85%. Accordingly, a rapid reliable point-of-care immunologic test is still a major challenge. It should be recognized that even culture methodologies do not confirm a causal relationship between the presence of *Candida* at a mucosal site and the associated pathology. Accordingly, positive cultures always require clinical correlation. Finally, for almost 5 years, PCR methodology has been available to identify the presence of *Candida* at mucosal sites. Numerous commercial operations are now available for diagnosis using nucleic acid amplification methodologies. As for cultures, the results are usually available after several hours and more frequently after several days. Accordingly, a rapid point-of-care test continues to be elusive. Even when available, it is unclear to what extent a PCR test is of value in diagnosing a mucosal infection. The potential for the enhanced sensitivity is enormous. It will be important to study PCR technologies in the clinical context before determining whether they offer any advantage over conventional methodologies. It is noteworthy that given the fact that *Candida* may be present as a colonizer (commensal) as well as a pathogen at a mucosal site, no tests are available that specifically identify *Candida* in a pathological phase using methodologies that would recognize virulence factors expressed during the pathological state. A point-of-care, inexpensive test that

not only identifies *Candida* but also indicates its pathogenic role would be an enormous advantage.

New Treatments

As mentioned above, the pipeline of new antifungals for mucosal candidiasis is almost empty and has been for several years. Apart from the introduction of oral posaconazole and a bioadhesive form of miconazole for OC, no new antifungal agents have been introduced for OC. Likewise, there have been no therapeutic innovations for vaginal candidiasis. Accordingly, newer agents are needed which are not fungistatic but rapidly fungicidal, which would be more effective in achieving eradication of the organism rather than allowing persistence of low numbers of the organism at an anatomical site that may be the site of the host defect. What is needed are new agents that are fungicidal, inexpensive, and broad spectrum. Topical agents may be easier to develop due to fewer side effects and complications than with systemic therapy.

Non-*C. albicans Candida* Species

In all forms of candidiasis, the overwhelming majority of infections are caused by *C. albicans* and the non-*C. albicans Candida* species are responsible for disease in only profoundly immunosuppressed patients heavily exposed to azole agents. Under these circumstances, *Candida glabrata* and, rarely, other species may be responsible for disease. In fact, non-*C. albicans Candida* species are now responsible for an increased proportion of patients with chronic and refractory disease. Many of these *Candida* species, including *Candida glabrata* and *Candia krusei*, are resistant to fluconazole. Accordingly, therapeutic agents, both topical and oral or systemic, that are active against non-*C. albicans Candida* species are urgently needed. Flucytosine is highly effective against *Candida glabrata* but is not widely available in oral form, and a topical formulation is not commercially available.

Candida Resistance

As mentioned above, *C. albicans* resistance to azole agents is rarely seen with oral disease other than in patients who are profoundly immunosuppressed. The availability of posaconazole offers an advantage in refractory OC. Similarly, the parenteral echinocandins are extremely useful for azole-resistant *C. albicans* EC. *C. albicans* azole resistance has been rare in vaginal candidiasis (48). Over the last 3 to 5 years, an increased number of women with azole-resistant organisms have been reported. Many of these isolates are cross resistant to other imidazoles and triazoles. In vaginal candidiasis, *C. albicans* resistance to these agents creates enormous problems, and frequently topical boric acid constitutes the only alternative agent. The need for effective topical or systemic agents active against azole-resistant *C. albicans* is evident. There is little evidence that oral voriconazole or posaconazole is effective for these fluconazole-resistant vaginal isolates.

Pathogenesis

In spite of progress in understanding the pathogenesis of mucosal candidiasis, there continues to be significant defects in our knowledge of normal mucosal host defense mechanisms, both innate and acquired, effective in preventing symptomatic mucosal candidiasis. This applies to both OC and vaginal candidiasis, although the relative importance of each component may differ. We still have a poor understanding of susceptibility to vaginal candidiasis in otherwise healthy women. The recent reports of host genes responsible for enhanced *Candida* vaginal colonization and the loss of tolerance on the part of the local immune system may well explain susceptibility to recurrent vaginal candidiasis.

Vaccine Development

There have been impressive studies using animal models that would suggest that there is a role for vaccine development for mucosal candidiasis. Clearly the benefits of a vaccine may also have an impact on systemic candidiasis in high-risk populations. Nevertheless, given the frequency worldwide of both OC and vaginal candidiasis, these would appear to be natural targets for the development of an effective vaccine that would decrease the frequency of recurrent vaginal candidiasis in (i) women so predisposed and (ii) women at risk of even occasional episodes of vaginal candidiasis. Needless to say, the commercial implications are just enormous. OC, on the other hand, tends to occur predominantly in immunocompromised patients with recognizable risk factors, but nevertheless, therapeutic failures in this area similarly indicate that a vaccine might be useful.

REFERENCES

1. **Achkar, J. M., and B. C. Fries.** 2010. *Candida* infections of the genitourinary tract. *Clin. Microbiol. Rev.* **23:**253–273.
2. **Ally, R., D. Schürmann, W. Kreisel, G. Carosi, K. Aguirrebengoa, B. Dupont, M. Hodges, P. Troke, A. J. Romero, and the Esophageal Candidiasis Study Group**. 2001. A randomized, double-blind, double-dummy, multicenter trial of voriconazole and fluconazole in the treatment of esophageal candidiasis in immunocompromised patients. *Clin. Infect. Dis.* **33:**1447–1454.
3. **Arathoon, E. G., E. Gotuzzo, L. M. Noriega, R. S. Berman, M. J. DiNubile, and C. A. Sable.** 2002. Randomized, double-blind, multicenter study of caspofungin versus amphotericin B for treatment of oropharyngeal and esophageal candidiasis. *Antimicrob. Agents Chemother.* **46:**451–457.
4. **Bensadoun, R. J., J. Daoud, B. El Gueddari, L. Bastit, R. Gourmet, A. Rosikon, C. Allavena, P. Céruse, G. Calais, and P. Attali.** 2008. Comparison of the efficacy and safety of miconazole 50-mg mucoadhesive buccal tablets with miconazole 500-mg gel in the treatment of oropharyngeal candidiasis: a prospective, randomized, single-blind, multicenter, comparative, phase III trial in patients treated with radiotherapy for head and neck cancer. *Cancer* **112:**204–211.
5. **Cassone, A., F. De Bernardis, and G. Santoni.** 2007. Anticandidal immunity and vaginitis: novel opportunities for immune intervention. *Infect. Immun.* **75:**4675–4686.
6. **Challacombe, S. J., and J. R. Naglik.** 2006. The effects of HIV infection on oral mucosal immunity. *Adv. Dent. Res.* **19:**29–35.
7. **Cheng, S. C., L. Y. Chai, L. A. Joosten, A. Vecchiarelli, B. Hube, J. W. Van Der Meer, B. J. Kullberg, and M. G. Netea.** 2010. *Candida albicans* releases soluble factors that potentiate cytokine production by human cells through a protease-activated receptor 1- and 2-independent pathway. *Infect. Immun.* **78:**393–399.
8. **de Wet, N., A. Llanos-Cuentas, J. Suleiman, E. Baraldi, E. F. Krantz, M. Della Negra, and H. Diekmann-Berndt.** 2004. A randomized, double-blind, parallel-group, dose-response study of micafungin compared with fluconazole for the treatment of esophageal candidiasis in HIV-positive patients. *Clin. Infect. Dis.* **39:**842–849.
9. **Dinubile, M. J., R. J. Lupinacci, R. S. Berman, and C. A. Sable.** 2002. Response and relapse rates of candidal

esophagitis in HIV-infected patients treated with caspofungin. *AIDS Res. Hum. Retrovir.* **18:**903–908.
10. **Donders, G. G., O. Babula, G. Bellen, I. M. Linhares, and S. S. Witkin.** 2008. Mannose-binding lectin gene polymorphism and resistance to therapy in women with recurrent vulvovaginal candidiasis. *BJOG* **115:**1225–1231.
11. **Duerr, A., M. F. Sierra, J. Feldman, L. M. Clarke, I. Ehrlich, and J. DeHovitz.** 1997. Immune compromise and prevalence of *Candida* vulvovaginitis in human immunodeficiency virus-infected women. *Obstet. Gynecol.* **90:** 252–256.
12. **Ferwerda, B., G. Ferwerda, T. S. Plantinga, J. A. Willment, A. B. van Spriel, H. Venselaar, C. C. Elbers, M. D. Johnson, A. Cambi, C. Huysamen, L. Jacobs, T. Jansen, K. Verheijen, L. Masthoff, S. A. Morré, G. Vriend, D. L. Williams, J. R. Perfect, L. A. Joosten, C. Wijmenga, J. W. van der Meer, G. J. Adema, B. J. Kullberg, G. D. Brown, and M. G. Netea. 2009**. Human dectin-1 deficiency and mucocutaneous fungal infections. *N. Engl. J. Med.* **361:**1760–1767.
13. **Fichtenbaum, C. J., R. Zackin, N. Rajicic, W. G. Powderly, L. J. Wheat, B. S. Zingman, and Adult AIDS Clinical Trials Group Study Team 295.** 2000. Amphotericin B oral suspension for fluconazole-refractory oral candidiasis in persons with HIV infection. *AIDS* **14:**845–852.
14. **Fidel, P. L., Jr.** 2006. *Candida*-host interactions in HIV disease: relationships in oropharyngeal candidiasis. *Adv. Dent. Res.* **19:**80–84.
15. **Gantner, B. N., R. M. Simmons, S. J. Canavera, S. Akira, and D. M. Underhill.** 2003. Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2. *J. Exp. Med.* **197:**1107–1117.
16. **Giraldo, P. C., O. Babula, A. K. Gonçalves, I. M. Linhares, R. L. Amaral, W. J. Ledger, and S. S. Witkin.** 2007. Mannose-binding lectin gene polymorphism, vulvovaginal candidiasis, and bacterial vaginosis. *Obstet. Gynecol.* **109:**1123–1128.
17. **Goldman, M., G. A. Cloud, K. D. Wade, A. C. Reboli, C. J. Fichtenbaum, R. Hafner, J. D. Sobel, W. G. Powderly, T. F. Patterson, L. J. Wheat, D. K. Stein, W. E. Dismukes, S. G. Filler, and AIDS Clinical Trials Group Study Team 323/Mycoses Study Group Study Team 40.** 2005. A randomized study of the use of fluconazole in continuous versus episodic therapy in patients with advanced HIV infection and a history of oropharyngeal candidiasis. *Clin. Infect. Dis.* **41:**1473–1480.
18. **Gonsalves, W. C., A. S. Wrightson, and R. G. Henry.** 2008. Common oral conditions in older persons. *Am. Fam. Physician* **78:**845–852.
19. **Gow, N. A., M. G. Netea, C. A. Munro, G. Ferwerda, S. Bates, H. M. Mora-Montes, L. Walker, T. Jansen, L. Jacobs, V. Tsoni, G. D. Brown, F. C. Odds, J. W. Van der Meer, A. J. Brown, and B. J. Kullberg.** 2007. Immune recognition of *Candida albicans* beta-glucan by dectin-1. *J. Infect. Dis.* **196:**1565–1571.
20. **Hancock, P. J., J. B. Epstein, and G. R. Sadler.** 2003. Oral and dental management related to radiation therapy for head and neck cancer. *J. Can. Dent. Assoc.* **69:** 585–590.
21. **Harriott, M. M., E. A. Lilly, T. E. Rodriguez, P. L. Fidel, Jr., and M. C. Noverr.** 2010. *Candida albicans* forms biofilms on the vaginal mucosa. *Microbiology* **156:** 3635–3644.
22. **Henriques, M., J. Azeredo, and R. Oliveira.** 2006. *Candida* species adhesion to oral epithelium: factors involved and experimental methodology used. *Crit. Rev. Microbiol.* **32:**217–226.
23. **Hurley, R., and J. De Louvois.** 1979. Candida vaginitis. *Postgrad. Med. J.* **55:**645–647.
24. **Kartsonis, N., M. J. DiNubile, K. Bartizal, P. S. Hicks, D. Ryan, and C. A. Sable.** 2002. Efficacy of caspofungin in the treatment of esophageal candidiasis resistant to fluconazole. *J. Acquir. Immune Defic. Syndr.* **31:**183–187.
25. **Kirkpatrick, C. H.** 1994. Chronic mucocutaneous candidiasis. *J. Am. Acad. Dermatol.* **31:**S14–S17.
26. **Koks, C. H., P. L. Meenhorst, A. Bult, and J. H. Beijnen.** 2002. Itraconazole solution: summary of pharmacokinetic features and review of activity in the treatment of fluconazole-resistant oral candidosis in HIV-infected persons. *Pharmacol. Res.* **46:**195–201.
27. **Krause, D. S., A. E. Simjee, C. van Rensburg, J. Viljoen, T. J. Walsh, B. P. Goldstein, M. Wible, and T. Henkel.** 2004. A randomized, double-blind trial of anidulafungin versus fluconazole for the treatment of esophageal candidiasis. *Clin. Infect. Dis.* **39:**770–775.
28. **Laudenbach, J. M., and J. B. Epstein.** 2009. Treatment strategies for oropharyngeal candidiasis. *Expert Opin. Pharmacother.* **10:**1413–1421.
29. **Lev-Sagie, A., P. Nyirjesy, N. Tarangelo, A. M. Bongiovanni, C. Bayer, I. M. Linhares, P. C. Giraldo, W. J. Ledger, and S. S. Witkin.** 2009. Hyaluronan in vaginal secretions: association with recurrent vulvovaginal candidiasis. *Am. J. Obstet. Gynecol.* **206:**e1–e5.
30. **Lynch, D. P.** 1994. Oral candidiasis. History, classification, and clinical presentation. *Oral Surg. Oral Med. Oral Pathol.* **78:**189–193.
31. **Marot-Leblond, A., S. Nail-Billaud, F. Pilon, B. Beucher, D. Poulain, and R. Robert.** 2009. Efficient diagnosis of vulvovaginal candidiasis by use of a new rapid immunochromatography test. *J. Clin. Microbiol.* **47:**3821–3825.
32. **McClelland, R. S., B. A. Richardson, W. M. Hassan, S. M. Graham, J. Kiarie, J. M. Baeten, K. Mandaliya, W. Jaoko, J. O. Ndinya-Achola, and K. K. Holmes.** 2009. Prospective study of vaginal bacterial flora and other risk factors for vulvovaginal candidiasis. *J. Infect. Dis.* **199:** 1883–1890.
33. **Milanese, M., L. Segat, F. De Seta, D. Pirulli, A. Fabris, M. Morgutti, and S. Crovella.** 2008. MBL genetic screening in patients with recurrent vaginal infections. *Am. J. Reprod. Immunol.* **59:**146–151.
34. **Mohammad, A. R., P. J. Giannini, P. M. Preshaw, and H. Alliger.** 2004. Clinical and microbiological efficacy of chlorine dioxide in the management of chronic atrophic candidiasis: an open study. *Int. Dent. J.* **54:**154–158.
35. **Nacher, M., V. Vantilcke, F. Huber, A. Mahamat, M. El Guedj, A. Randrianjohany, E. Clyti, C. Aznar, B. Carme, and P. Couppié.** 2007. Increased incidence of mucosal candidiasis after HAART initiation: a benign form of immune reconstitution disease? *AIDS* **21:**2534–2536.
36. **Naglik, J. R., P. L. Fidel, Jr., and F. C. Odds.** 2008. Animal models of mucosal *Candida* infection. *FEMS Microbiol. Lett.* **283:**129–139.
37. **Netea, M. G., G. D. Brown, B. J. Kullberg, and N. A. Gow.** 2008. An integrated model of the recognition of *Candida albicans* by the innate immune system. *Nat. Rev. Microbiol.* **6:**67–78.
38. **Niimi, M., N. A. Firth, and R. D. Cannon.** 2010. Antifungal drug resistance of oral fungi. *Odontology* **98:**15–25.
39. **Nokta, M.** 2008. Oral manifestations associated with HIV infection. *Curr. HIV/AIDS Rep.* **5:**5–12.
40. **Nurbhai, M., J. Grimshaw, M. Watson, C. Bond, J. Mollison, and A. Ludbrook.** 2007. Oral versus intra-vaginal imidazole and triazole anti-fungal treatment of uncomplicated vulvovaginal candidiasis (thrush). *Cochrane Database Syst. Rev.* **17:**CD002845.
41. **Ohmit, S. E., J. D. Sobel, P. Schuman, A. Duerr, K. Mayer, A. Rompalo, R. S. Klein, and the HIV Epidemiology Research Study (HERS) Group.** 2003. Longitudinal

study of mucosal *Candida* species colonization and candidiasis among human immunodeficiency virus (HIV)-seropositive and at-risk HIV-seronegative women. *J. Infect. Dis.* **188:**118–127.

42. **Ozcan, K., M. Ilkit, A. Ates, A. Turac-Bicer, and H. Demirhindi.** 2010. Performance of chromogenic *Candida* agar and CHROMagar *Candida* in recovery and presumptive identification of monofungal and polyfungal vaginal isolates. *Med. Mycol.* **48:**29–34.

42a. **Pappas, P G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, J. D Sobel, and the Infectious Diseases Society of America.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.

43. **Pirotta, M. V., and S. M. Garland.** 2006. Genital *Candida* species detected in samples from women in Melbourne, Australia, before and after treatment with antibiotics. *J. Clin. Microbiol.* **44:**3213–3217.

44. **Rosenbach, A., D. Dignard, J. V. Pierce, M. Whiteway, and C. A. Kumamoto.** 2010. Adaptations of *Candida albicans* for growth in the mammalian intestinal tract. *Eukaryot. Cell* **9:**1075–1086.

45. **Schuman, P., L. Capps, G. Peng, J. Vazquez, W. el-Sadr, A. I. Goldman, B. Alston, C. L. Besch, A. Vaughn, M. A. Thompson, M. N. Cobb, T. Kerkering, and J. D. Sobel.** 1997. Weekly fluconazole for the prevention of mucosal candidiasis in women with HIV infection. A randomized, double-blind, placebo-controlled trial. Terry Beirn Community Programs for Clinical Research on AIDS. *Ann. Intern. Med.* **126:**689–696.

46. **Sha, B. E., M. R. Zariffard, Q. J. Wang, H. Y. Chen, J. Bremer, M. H. Cohen, and G. T. Spear.** 2005. Female genital-tract HIV load correlates inversely with *Lactobacillus* species but positively with bacterial vaginosis and *Mycoplasma hominis*. *J. Infect. Dis.* **191:**25–32.

47. **Skiest, D. J., J. A. Vazquez, G. M. Anstead, J. R. Graybill, J. Reynes, D. Ward, R. Hare, N. Boparai, and R. Isaacs.** 2007. Posaconazole for the treatment of azole-refractory oropharyngeal and esophageal candidiasis in subjects with HIV infection. *Clin. Infect. Dis.* **44:** 607–614.

48. **Sobel, J. D.** 2007. Vulvovaginal candidosis. *Lancet* **369:** 1961–1971.

49. **Sobel, J. D.** 1986. Recurrent vulvovaginal candidiasis: a prospective study of the efficacy of maintenance ketoconazole therapy. *N. Engl. J. Med.* **315:**1455–1458.

50. **Sobel, J. D., S. Faro, R. W. Force, B. Foxman, W. J. Ledger, P. R. Nyirjesy, B. D. Reed, and P. R. Summers.** 1998. Vulvovaginal candidiasis: epidemiologic, diagnostic and therapeutic considerations. *Am. J. Obstet. Gynecol.* **178:**203–211.

51. **Sobel, J. D., P. S. Kapernick, M. Zervos, B. D. Reed, T. Hooton, D. Soper, P. Nyirjesy, M. W. Heine, J. Willems, H. Panzer, and H. Wittes.** 2001. Treatment of complicated *Candida* vaginitis: comparison of single and sequential doses of fluconazole. *Am. J. Obstet. Gynecol.* **185:**363–369.

52. **Sobel, J. D., W. Chaim, V. Nagappan, and D. Leaman.** 2003. Treatment of vaginitis caused by *Candida glabrata*: use of boric acid and flucytosine. *Am. J. Obstet. Gynecol.* **189:**1297–1300.

53. **Sobel, J. D., H. C. Wiesenfeld, M. Martens, P. Danna, T. M. Hooton, A. Rompalo, M. Sperling, C. Livengood, B. Horowitz, J. Von Thron, L. Edwards, H. Panzer, and T. C. Chu.** 2004. A randomized, placebo-controlled trial of maintenance fluconazole therapy for management of recurrent vulvovaginal candidiasis. *N. Engl. J. Med.* **351:**876–883.

54. **Soysa, N. S., L. P. Samaranayake, and A. N. Ellepola.** 2008. Antimicrobials as a contributory factor in oral candidosis—a brief overview. *Oral Dis.* **14:**138–143.

55. **Trama, J. P., M. E. Adelson, I. Raphaelli, S. M. Stemmer, and E. Mordechai.** 2005. Detection of *Candida* species in vaginal samples in a clinical laboratory setting. *Infect. Dis. Obstet. Gynecol.* **13:**63–67.

56. **Vazquez, J. A., and J. D. Sobel.** 2002. Mucosal candidiasis. *Infect. Dis. Clin. N. Am.* **16:**793–820.

57. **Vazquez, J. A., D. J. Skiest, H. Tissot-Dupont, J. L. Lennox, N. Boparai, and R. Isaacs.** 2007. Safety and efficacy of posaconazole in the long-term treatment of azole-refractory oropharyngeal and esophageal candidiasis in patients with HIV infection. *HIV Clin. Trials* **8:**86–97.

58. **Vazquez, J. A., J. A. Schranz, K. Clark, B. P. Goldstein, A. Reboli, and C. Fichtenbaum.** 2008. A phase 2, open-label study of the safety and efficacy of intravenous anidulafungin as a treatment for azole-refractory mucosal candidiasis. *J. Acquir. Immune Defic. Syndr.* **48:**304–309.

59. **Villanueva, A., E. Gotuzzo, E. G. Arathoon, L. M. Noriega, N. A. Kartsonis, R. J. Lupinacci, J. M. Smietana, M. J. DiNubile, and C. A. Sable.** 2002. A randomized double-blind study of caspofungin versus fluconazole for the treatment of esophageal candidiasis. *Am. J. Med.* **113:**294–299.

60. **Villar, C. C., and A. Dongari-Bagtzoglou.** 2008. Immune defense mechanisms and immunoenhancement strategies in oropharyngeal candidiasis. *Expert Rev. Mol. Med.* **10:**e29.

61. **Weerasuriya, N., and J. Snape.** 2008. Oesophageal candidiasis in elderly patients: risk factors, prevention and management. *Drugs Aging* **25:**119–130.

62. **Werneck-Silva, A. L., and I. B. Prado.** 2009. Role of upper endoscopy in diagnosing opportunistic infections in human immunodeficiency virus-infected patients. *World J. Gastroenterol.* **15:**1050–1056.

63. **White, M. H.** 1996. Is vulvovaginal candidiasis an AIDS-related illness? *Clin. Infect. Dis.* **22:**S124–S127.

64. **Wilcox, C. M., R. O. Darouiche, L. Laine, B. L. Moskovitz, I. Mallegol, and J. Wu.** 1997. A randomized, double-blind comparison of itraconazole oral solution and fluconazole tablets in the treatment of esophageal candidiasis. *J. Infect. Dis.* **176:**227–232.

65. **Witkin, S. S.** 1991. Immunologic factors influencing susceptibility to recurrent candidal vaginitis. *Clin. Obstet. Gynecol.* **34:**662–668.

66. **Young, G. L., and D. Jewell.** 2001. Topical treatment for vaginal candidiasis (thrush) in pregnancy. *Cochrane Database Syst. Rev.* **4:**CD000225.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy
©2012 ASM Press, Washington, DC
10.1128/9781555817176.ch27

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Systemic Candidiasis: Candidemia and Deep-Organ Infections

CORNELIUS J. CLANCY AND M. HONG NGUYEN

Candida species are major fungal pathogens of humans, causing a diverse range of mucosal and more deeply invasive diseases (mucosal and invasive candidiasis, respectively) in both immunocompromised and immunocompetent hosts (38, 121, 149). Most notable among the various types of invasive candidiasis is candidemia, which is the fourth most common bloodstream infection in the United States and is associated with attributable mortality in some reports that exceeds 40% (51). In addition to candidemia, invasive candidiasis comprises a wide spectrum of nonmucosal diseases, reflecting the remarkable ability of *Candida* species to infect virtually any organ after gaining access to the bloodstream or, less commonly, upon being directly introduced as a result of medical procedures, trauma, etc. End-organ diseases, therefore, may be encountered in the presence or absence of documented candidemia. When occurring as a complication of hematogenous dissemination, invasive candidiasis often involves multiple organs, as detailed below. The typical histopathological findings are diffuse microabscesses and small macroabscesses with acute suppurative and granulomatous reactions (28). Larger macroabscesses may be observed, particularly in the liver and spleen in patients receiving chemotherapy for malignancies (28). In many cases of invasive candidiasis, the absence of candidemia at the time of diagnosis reflects the poor sensitivity of blood cultures for *Candida* species rather than the lack of bloodstream organisms (32). By the same token, *Candida* species can seed end organs cryptically as a result of unrecognized hematogenous dissemination. In this event, diseases such as *Candida* osteomyelitis and ophthalmitis may not manifest until days or weeks after bloodstream infections have cleared. Therefore, even in the absence of positive blood cultures, the diagnosis of invasive candidiasis at a tissue site should prompt consideration of other organ involvement.

In this chapter, we review the clinical characteristics and specific treatment recommendations for the various types of invasive candidiasis, beginning with candidemia. In our usage, candidemia means bloodstream candidiasis. Disseminated (systemic) candidiasis refers to end-organ disease stemming from hematogenous seeding, since any bloodborne *Candida* infection may infect the organs through which it circulates. After our discussion of candidemia, we address specific organ infections separately. Elsewhere in this book, there are detailed chapters devoted to other important clinical topics, including mucosal candidiasis, basic microbiology and distinctions among *Candida* species, epidemiology and ecology, pathogenesis, diagnostic tests, and antifungal drugs. Therefore, we do not address these topics in depth. This chapter is designed for a readership of diverse backgrounds, with an eye towards the many nonclinician researchers and non-infectious-disease specialists who utilize Candida *and Candidiasis* as a reference text. We pay particular attention to the thought processes used by clinicians, the challenges they face in making clinical decisions, and the typical time line of events during the management of invasive candidiasis.

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CANDIDEMIA

Clinical Characteristics and Challenges

The emergence of candidiemia as a major health care problem over recent decades is a result of advances in medical care, as at-risk patient populations have increased dramatically due to the widespread use of immunosuppressive drugs and developments in interventional techniques and intensive care management (43, 92, 116, 155, 156, 159, 175). The risk factors for candidemia are numerous and well described, including neutropenia, receipt of immunosuppressive drugs and other conditions associated with suppressed immune function, receipt of broad-spectrum antibiotics, use of central venous catheters and total parenteral nutrition, gastrointestinal surgery, burn injuries, and hemodialysis and other forms of renal replacement therapy (13, 73, 102, 103, 124, 127, 170). While *Candida* species generally are considered opportunistic pathogens, it is important to recognize that candidemia is encountered increasingly in patients who are not neutropenic or otherwise profoundly immunosuppressed. In part, this trend reflects the availability of more and better-tolerated antifungal agents and widespread use of

prophylactic or empirical treatment regimens among classic at-risk groups, such as patients with acute leukemia and stem cell or solid-organ transplant recipients. Indeed, empirical antifungal therapy in persistently febrile and neutropenic patients from these risk groups was shown to reduce invasive fungal infections and mortality in a long series of clinical trials (34, 125). Since such neutropenic patients are at risk for diverse fungal diseases, empirical therapy is directed against moulds like *Aspergillus* species, as well as *Candida*.

As invasive candidiasis is less commonly diagnosed in the classic high-risk groups, it is encountered more often among other critically ill patients and persons with indwelling vascular access devices. In fact, the majority of cases of candidemia currently occur in intensive care units or on general-surgery floors (83, 175). Recognizing the importance of candidemia in such populations, intensivists, surgeons, and other clinicians caring for these patients face several major challenges at present. As mentioned above, a major challenge is presented by the inadequacy of existing diagnostic tests. It is clear that the prompt institution of antifungal therapy is a major determinant of outcome among patients with candidemia (44, 93), but blood cultures, the current "gold standard" test, are positive in only 50 to 70% of cases (32). Even when blood cultures reveal *Candida*, they often do so late in the disease course (32). As a result, a minority of patients who have candidemia are diagnosed in time to receive optimal antifungal therapy (28). Given the mortality rates cited above, this situation creates pressure on clinicians to initiate empirical antifungal therapy to patients in whom candidemia is suspected but not diagnosed. Once antifungal therapy is started empirically, the inadequacy of diagnostic tests also limits the ability of clinicians to discontinue these agents. The result, not uncommonly, is prolonged courses of empirical treatment, which may contribute to the emergence of antifungal drug resistance. This problem is particularly true for agents such as fluconazole and the echinocandins, which are well tolerated and associated with lower rates of toxicity than earlier options such as amphotericin B products.

As clinicians make their decisions about whether to institute antifungal therapy in the absence of a definitive diagnosis of candidemia, they confront two additional, interrelated challenges. First, the clinical manifestations of candidemia are nonspecific, ranging from a lack of signs and symptoms to septic shock. Most commonly, patients present with fevers of acute or insidious onset, which are often present despite antibiotic therapy and may be continuous or intermittent (40). In many cases, particularly of catheter-associated candidemia, patients may have a single temperature spike, which resolves in the absence of treatment by the time the diagnosis is made. In this setting, it is imperative to understand that candidemia remains a serious disease, as dissemination of *Candida* species to various organs is well described despite the absence of signs or symptoms (40, 70). Septic shock due to *Candida* species, on the other hand, is indistinguishable from sepsis due to other causes, manifesting as fever, hypotension and cardiovascular instability, and multisystem organ dysfunction. Distinctive skin lesions of candidemia are encountered in up to 10% of patients, but they are most likely to occur in neutropenic patients and rare in nonneutropenic patients (40). Therefore, given the wide range of possible signs and symptoms associated with candidemia and the numerous alternative infectious and noninfectious etiologies that may explain them, clinicians cannot rely upon clinical findings or standard laboratory test results to identify patients who may benefit from empirical therapy.

The difficulties are compounded by the fact that risk factors for candidemia, although well recognized, are so common among patients in intensive care units that it is difficult for clinicians to reliably identify the highest-risk subgroups of patients to target empirically. As described in other chapters of this book, the development of clinical prediction scores for candidemia based on risk factors, sites of *Candida* colonization, and new diagnostic markers such as beta-glucan detection is an area of robust investigation (74, 107, 108, 110, 112, 114). In general, prediction models that do not incorporate diagnostic markers are characterized by high specificity but low sensitivity (115, 134). Consequently, a positive result is highly likely to identify a patient with candidemia, but a large percentage of patients who will develop candidemia are not identified. Sensitivity with beta-glucan detection is as high as 80 to 90%, but specificity with widespread use is unclear (107, 108, 110). Cost-benefit analyses suggest that accurately identifying a population with a 15 to 20% pretest likelihood of candidemia would support an empirical intervention strategy (see detailed discussion in chapter 28). Until therapeutic interventions based on such approaches are validated, however, clinicians are left with the choices of (i) waiting to treat patients until a definitive diagnosis is made, most commonly by blood cultures, accepting that a number of patients with candidemia will not be identified or will be identified too late to benefit from therapy; or (ii) empirically treating a large number of patients who do not have disease or would never develop disease in order to treat the relatively small percentage that would benefit from empirical therapy, accepting the financial costs and potential for drug toxicity and side effects, drug-drug interactions, selection for resistance, and impact on hospital microbial ecology.

Treatment

Consensus practice guidelines have been developed for candidemia and other types of candidiasis, to which readers are referred for detailed discussions of treatment recommendations and options (115, 134). Below we review empirical therapy and treatment of proven candidemia in both nonneutropenic and neutropenic hosts.

Empirical Therapy in Nonneutropenic Hosts

As alluded to in the preceding section, the clinical benefits and indications for initiating empirical therapy remain undefined (123, 143, 145). In the absence of clear-cut criteria, therefore, clinicians are left to their best judgment in specific cases, guided by their understanding of the patient's condition and local epidemiology, disease incidence, microbiology, and susceptibility patterns. In general, empirical therapy should be considered for critically ill, nonneutropenic patients with fever or other signs and symptoms suggestive of a systemic infection (e.g., hemodynamic instability, changes in mental status, elevated white blood cell count and/or immature forms of white blood cells in the peripheral blood, etc.), risk factors for candidemia, and no alternative diagnosis that explains the findings. For patients who are hemodynamically unstable, have been previously exposed to fluconazole or other azole agents, or are known to be colonized with azole-resistant *Candida* species, echinocandins are the agents of first choice (115). Dosing recommendations are caspofungin (70-mg loading dose and then 50 mg daily), anidulafungin (200-mg loading dose and then 100 mg daily), or micafungin (100 mg daily). The echi-

nocandin agents should be considered clinically equivalent, with specific choices usually dictated by the institutional formulary. Amphotericin B products are acceptable alternatives to the echinocandins, having the advantage of powerful fungicidal activity. Dosing recommendations are amphotericin B deoxycholate (0.5 to 1.0 mg/kg of body weight daily) and lipid formulation amphotericin B (3 to 5 mg/kg daily). Amphotericin B agents have largely been superseded by the echinocandins, due to drug toxicity considerations.

For nonneutropenic patients who are less critically ill, have not been previously exposed to an azole, and are not colonized by species with reduced azole susceptibility, fluconazole (800-mg [12-mg/kg] loading dose and then 400 mg [6 mg/kg] daily) is generally the front-line agent and echinocandins are the backup option. In institutions with particularly high rates of candidiasis caused by species that may be less susceptible to azole agents, such as *Candida krusei* or *Candida glabrata*, echinocandins may be used initially as empirical therapy. Even in such centers, however, azole-resistant candidemia is unlikely in the absence of prior azole exposure, and fluconazole remains an acceptable up-front choice. If an echinocandin is chosen as initial therapy in these settings, a change to fluconazole should be made once a non-*C. krusei* or non-*C. glabrata* species is identified or azole susceptibility is confirmed.

Empirical Therapy in Neutropenic Hosts

Unlike the case with nonneutropenic hosts, the use of empirical antifungal therapy among febrile neutropenic hosts is well established and validated by outcome data in clinical trials (34, 125). As mentioned above, the best regimens in this setting are active against both *Candida* and moulds. Front-line options include liposomal amphotericin B, voriconazole (6 mg/kg twice daily for two doses and then 3 mg/kg twice daily), or caspofungin. Liposomal amphotericin B is favored over amphotericin B deoxycholate because of its improved toxicity profile and greater success in preventing breakthrough infections (162), and over amphotericin B lipid complex and amphotericin B colloidal dispersion because of fewer infusion-related reactions (171, 173). Voriconazole is effective against aspergillosis and candidemia and prevents breakthrough fungal infections in neutropenic hosts (68, 163). The role of posaconazole in empirical regimens is undefined. Caspofungin is as effective empirically as liposomal amphotericin B and is better tolerated (164). Micafungin and anidulafungin, on the other hand, have not been studied as empirical therapy in the setting of neutropenia. Fluconazole and itraconazole (200 mg [3 mg/kg] twice daily) are alternatives to the front-line agents but are limited by a lack of activity against moulds and variable absorption and gastrointestinal toxicity, respectively (15, 82, 158, 174). In general, azoles should not be used for empirical therapy in patients who received azole prophylaxis previously.

Treatment of Proven Candidemia

Under usual circumstances, clinicians first become aware of candidemia due to a positive blood culture. When automated laboratory systems identify microbial growth within a blood culture bottle, clinical microbiology personnel typically perform a Gram stain to identify the morphology of the responsible organism. At this point, therefore, clinicians are aware that a yeast is isolated, but they must wait for subcultures to grow before formal species identification is completed. During this window period, which may last 48 h or longer, the initial therapeutic decisions are undertaken. Techniques to speed this process, such as nucleic acid amplification, fluorescent in situ hybridization, and use of chromogenic agar, have been developed and are increasingly incorporated into clinical practice to more rapidly tailor antifungal therapy. In considering the treatment of proven candidemia, therefore, it is useful to divide management decisions into those made prior to the identification of *Candida* species and those made afterwards.

At this point, it is important to point out that a central tenet of infectious-disease clinical practice is that any positive blood culture for *Candida* must be treated, even if a patient is asymptomatic and the infection is clearly associated with an intravenous catheter that can be removed (28, 29, 131). In other words, there are no such clinical entities as *Candida* catheter colonization, contamination, or transient bloodstream infections. Indeed, mortality rates among candidemic patients who are not treated with an antifungal approach 75% (40, 70), and hematogenous seeding of end organs occurs in up to 35% of patients (66, 70). In keeping with our earlier discussion of the difficulties clinicians face in identifying patients with candidemia based on signs or symptoms, clinical judgment also fails to accurately identify those patients with candidemia who may develop complications or die (10, 12).

Treatment of Candidemia Prior to Identification of *Candida* Species

In general, the initial treatment of nonneutropenic patients who have been diagnosed with candidemia follows the principles outlined above for empirical therapy in this population. Until a species is known by the clinician, the choice of therapy is on most occasions between fluconazole and an echinocandin. This decision is shaped by factors such as the overall condition of the patient, immune function, institutional microbiology and antifungal susceptibility patterns, prior receipt of antifungal agents, and evidence for concomitant *Candida* infections of end organs. As indicated above, factors favoring fluconazole include milder disease, more intact immune function, low institutional incidence or low clinical likelihood of azole-resistant species, no prior azole exposure, and a lack of end-organ diseases such as endocarditis or meningitis for which fungicidal agents would be preferable. Under such conditions, there is ample evidence for the efficacy of fluconazole (68, 130, 132, 133). At least initially, parenteral fluconazole therapy is preferable to oral therapy in most patients, to ensure compliance and delivery of drug. Although an echinocandin was superior to fluconazole as initial therapy against candidemia in a recent randomized study (130), these agents are best reserved for more seriously ill patients and those for whom the above criteria for fluconazole are not fulfilled. In the event of serious end-organ diseases like cardiac or central nervous system infections, lipid formulations of amphotericin B, the most fungicidal of options, may be chosen over echinocandins. The principles of managing specific types of invasive candidiasis are discussed in greater detail below.

There are fewer published data for the management of candidemia in neutropenic hosts, who are less frequently enrolled in clinical trials. Following the general approach mentioned in the preceding paragraph, an echinocandin is usually the initial therapy for patients with positive blood cultures who are neutropenic (6, 12, 69, 92, 117, 130). Over the years, however, the greatest experience in this population has been with amphotericin B products, which represent an alternative to the echinocandins. Based on the more extensive experience with amphotericin B products, some clinicians prefer these agents to the echinocandins,

particularly in clinically unstable patients. As in nonneutropenic hosts, lipid formulations of amphotericin B receive particular consideration in the treatment of serious end-organ infections. Fluconazole is less frequently used up front in neutropenic patients, although there is no conclusive evidence that it is less effective than the echinocandins or amphotericin B. Voriconazole offers an advantage by covering *C. krusei*, which is intrinsically resistant to fluconazole, and moulds, and it may be considered in settings where these pathogens are of concern (161). If mould coverage is not desired, however, the echinocandins are preferable choices due to less toxicity and more predictable pharmacokinetics. Provided that prompt antifungal therapy is initiated, outcomes among neutropenic patients with candidemia do not appear to be significantly worse than among nonneutropenic hosts. Mortality rates are typically higher among patients who remain persistently neutropenic and are greatly improved with recovery of neutrophil counts (5, 50, 60, 81, 159). The role of adjunctive therapy with recombinant granulocyte colony-stimulating factor to hasten the recovery of neutrophil counts is unclear, and such therapy cannot be recommended as routine practice. Clinicians may consider using this agent in selected patients with severe or refractory invasive candidiasis in the setting of persistent neutropenia (40).

Treatment of Candidemia after Identification of *Candida* Species

After the *Candida* species is identified, clinicians have a decision to make about continuing or altering the initial antifungal regimen. For nonneutropenic patients who are infected with species that are likely to be fluconazole susceptible such as *C. albicans*, *C. parapsilosis*, and *C. tropicalis*, and who have had a clinical response to initial treatment with an echinocandin or amphotericin B product, a switch to step-down therapy with fluconazole is advocated. For patients who are started on fluconazole, this agent can be continued. In patients who are clinically stable, oral fluconazole is considered after 3 to 5 days of parenteral therapy with an antifungal. Of course, many patients with candidemia will not be able to take oral medications or will have significant risk factors for poor gastrointestinal absorption, in which case parenteral therapy should be continued. The evidence supporting fluconazole step-down therapy and oral dosing is limited. While the approaches described are reasonable and reflect typical practices in treating nonneutropenic hosts, many clinicians are more conservative in switching from fungicidal agents or to oral therapy in neutropenic hosts. Conclusive data are not available, and in the end clinicians must make individualized decisions for each patient. Nevertheless, a transition to step-down fluconazole in clinically stable neutropenic patients who have responded to other agents is reasonable.

There are special considerations in managing candidemia due to one of several *Candida* species. For *C. parapsilosis*, fluconazole is favored over the echinocandins due to the decreased in vitro activity of the latter agents against this species (11, 161, 172). In especially severe cases or neutropenic hosts, an amphotericin B product may be considered. If patients were treated with an echinocandin prior to species identification and have improved clinically, it is acceptable to continue these agents, if so desired.

For candidemia due to *C. glabrata*, an echinocandin is preferred due to the possibility of decreased azole susceptibility. As with echinocandin therapy of *C. parapsilosis* infections, continuation of fluconazole against *C. glabrata* infections in the face of a clinical response to initial therapy is reasonable. Fluconazole can also be used as step-down therapy if the susceptibility of an infecting *C. glabrata* isolate is documented. Voriconazole is active against some, but not all, fluconazole-resistant *C. glabrata* isolates (36, 121). Therefore, it can be considered for step-down therapy once susceptibility is verified. For both *C. parapsilosis* treated with an echinocandin and *C. glabrata* treated with an azole, failure to sterilize blood cultures should prompt a change in therapy.

C. krusei is intrinsically resistant to fluconazole, and an echinocandin is preferred. Voriconazole is often active against *C. krusei* (36, 121), and if the susceptibility of a patient's isolate is confirmed, this agent can be considered. The other *Candida* species are extremely rare causes of human diseases; their typical antifungal susceptibility patterns are discussed in other chapters.

Candidemia in the absence of concomitant end-organ involvement is treated with antifungal agents for 2 weeks after the sterilization of blood cultures and the resolution of the signs and symptoms of disease (68, 69, 92, 115, 130, 132, 133). For neutropenic patients, the duration of treatment is similar, with the additional provision that antifungal agents are continued until after the resolution of neutropenia (69, 92, 115). To document sterilization of the bloodstream, follow-up blood cultures are obtained for all patients at least every other day until they are negative.

Other Aspects of Management

In the majority of cases of candidemia, particularly among cancer patients receiving chemotherapy, the portal of entry is believed to be the gastrointestinal tract rather than intravascular catheters (84, 124). Therefore, the issue of mandatory central venous catheter removal in the treatment of candidemia remains controversial. For nonneutropenic patients, data suggest that catheter removal is associated with decreased duration of candidemia and reduced mortality rates (63, 79, 103, 117, 131). For neutropenic patients, however, data are less compelling (106). Moreover, catheter removal is often problematic in these patients, as they frequently require long-term intravascular access. Despite the controversies, most clinicians and practice guidelines advocate catheter removal where possible (34, 79, 103, 131). In particular, candidemia due to *C. parapsilosis* is generally catheter related (5), favoring routine catheter removal.

Finally, all patients with candidemia require an ophthalmologic examination to exclude *Candida* endophthalmitis. This examination is best undertaken after the initiation of therapy and sterilization of the bloodstream, in order to minimize the possibility of subsequently seeding the eye. Similarly, any findings suggestive of an infectious process in other end organs should be investigated to exclude invasive candidiasis. The demonstration of end-organ disease in conjunction with candidemia will affect the duration of antifungal therapy and possibly have other management implications.

CANDIDA ENDOPHTHALMITIS

Endophthalmitis refers to inflammation of the internal coats of the eye. *Candida* endophthalmitis results most often from hematogenous dissemination and more rarely as a complication of cataract and other intraocular surgeries. *C. albicans* is the most common etiology, but other species are increasingly reported. In the past, almost 30% of nonneutropenic patients with candidemia were found to have evidence of

endophthalmitis if a thorough eye examination was performed (16). The incidence may be lower in more recent studies (27, 37, 135), perhaps as a result of increased empirical and prophylactic antifungal usage. In addition, the diagnosis is less commonly made in neutropenic hosts, as the characteristic white, cotton ball-like lesions are composed largely of neutrophils (54). While a diagnosis of *Candida* endophthalmitis should prompt an evaluation for disseminated candidiasis, half of patients have negative blood cultures (28). Patients may not have ocular symptoms, in which case they are diagnosed by routine fundoscopic examination in the setting of candidemia. If patients are symptomatic, typical findings include visual blurring, floating scotomas, and pain. Often, critically ill patients with candidemia are unable to complain of symptoms. Progression of lesions can result in permanent blindness.

The treatment of *Candida* endophthalmitis depends on the severity and location of the disease. Amphotericin B deoxycholate (0.7 to 1 mg/kg), combined with flucytosine (25 mg/kg administered four times daily), is used to treat advancing lesions or lesions threatening the macula. For patients with severe endophthalmitis and vitreitis, ophthalmologic consultation is recommended for consideration of partial vitrectomy and intravitreal antifungal therapy with amphotericin B deoxycholate, which can be sight saving (8, 87). Fluconazole (400 to 800 mg daily) is an acceptable alternative for less severe endophthalmitis. The recommended duration of therapy is at least 4 to 6 weeks and is determined by the stabilization or resolution of lesions as documented by repeated ophthalmologic examinations. Given the duration of therapy, step-down treatment with fluconazole is often used after an initial course of amphotericin B. The echinocandins are generally avoided due to their poor penetration into the eye.

CHRONIC DISSEMINATED CANDIDIASIS (HEPATOSPLENIC CANDIDIASIS)

Chronic disseminated candidiasis is a disease of neutropenic hosts, in which clinically inapparent seeding of the liver and spleen becomes evident as focal lesions upon recovery of neutrophil counts. In this regard, the disease may represent a form of immune reconstitution syndrome (71). Signs and symptoms include fever, abdominal pain, and hepatosplenomegaly, and laboratory tests may reveal liver function abnormalities (64). The diagnosis is suggested in the appropriate clinical setting by imaging studies. Although the characteristic lesions by computed tomography (CT) scan or magnetic resonance imaging (MRI) are often taken as diagnostic, a definitive microbiological diagnosis is recommended since other fungi can cause similar lesions (85). Blood cultures are occasionally, but not invariably, positive. For clinically stable patients, fluconazole is the treatment of choice (4, 64). For patients who are more seriously ill or for whom there is an inadequate response to fluconazole, the preferred alternative antifungal is an amphotericin B product, based on the long-standing experience with these agents (48, 154, 165). Echinocandins have also been used more recently (117, 151), as has voriconazole (111). After responses to amphotericin B or an echinocandin are achieved, step-down therapy with fluconazole is undertaken. Antifungal treatment is continued until lesions resolve or calcify, typically requiring weeks to months. If patients require ongoing or subsequent chemotherapy or stem cell transplantation, antifungal therapy is continued or reinitiated. In certain cases of large or refractory abscesses, splenectomy may be necessary. In keeping with the paradigm of immune reconstitution playing a role in the pathogenesis of disease, a study of adjuvant corticosteroid therapy reported favorable results in refractory cases (71). Recently published practice guidelines conclude that there is not enough evidence to recommend such therapy on a routine basis (115).

CANDIDIASIS OF THE ABDOMEN AND GASTROINTESTINAL TRACT

Chronic disseminated candidiasis may be considered a particular form of intra-abdominal candidiasis. The most common type of intra-abdominal fungal infection is *Candida* peritonitis, which most frequently occurs as a complication of peritoneal dialysis (3, 9, 176). In patients with peritonitis in the setting of peritoneal dialysis, the major risk factors are prior receipt of antibiotics and previous episodes of *Candida* peritonitis (49). Almost without exception, peritoneal dialysis-associated disease remains confined to the peritoneal cavity. Peritonitis occurs less often following gastrointestinal surgery, transplantation of an intra-abdominal organ, or perforation of an abdominal viscus (2, 17, 21, 49, 55, 150, 166). In the setting of gastrointestinal perforation, peritonitis may be complicated by disseminated candidiasis in 25% of cases (28). The diagnosis of peritonitis is made by culture or cytology of peritoneal dialysate (91).

Candida species also have been reported to infect several gastrointestinal organs, including the gallbladder and biliary tree, liver and spleen, and pancreas (26, 42, 57, 61). Ultrasound, CT, and MRI studies can visualize gastrointestinal abscesses, and laparoscopic techniques are useful in collecting cultures and making diagnoses. It is important that clinicians recognize that indwelling peritoneal and other intra-abdominal drains, similar to urinary catheters (see below), are very commonly colonized with *Candida* species after several days. Therefore, positive cultures in these settings are not necessarily diagnostic of disease, unlike the case for positive blood cultures. The distinction between colonization and disease in these cases is dependent upon clinical judgment and can be challenging even for experienced clinicians. *Candida* cultures obtained from an undrained peritoneum or abscess, or under sterile surgical conditions, on the other hand, are diagnostic of disease.

Candida peritonitis is treated most commonly with fluconazole, and amphotericin B is used as an alternative therapy for patients infected with fluconazole-resistant isolates (134). As in other types of candidiasis related to catheters or other foreign bodies, removal of peritoneal catheters is an important adjunct to antifungal agents (22, 91). Some cases of successful treatment with retained catheters are reported (136, 153), but relapses may occur. Local instillation of amphotericin B into the peritoneal cavity has been advocated in the past (28), but this approach is now discouraged due to peritoneal irritation and the availability of well-tolerated alternatives like fluconazole (39, 134). For other intra-abdominal infections, the choice between fluconazole and amphotericin B is at the discretion of the treating clinician. Both agents penetrate very well into the biliary tree, making them satisfactory for the treatment of cholecystitis. Biliary tract disease also requires drainage and decompression. In general, pancreatic abscesses and necrotizing pancreatitis due to *Candida* species require guided drainage or surgical resection.

The treatment of *Candida* species recovered from indwelling drains is generally not advocated (28). The topic,

however, is somewhat controversial (140), particularly since fluconazole penetrates into abdominal tissues well and is well tolerated. Benefits of antifungal therapy in this setting are not proven (28). Fluconazole prophylaxis to prevent *Candida* peritonitis was successful among high-risk surgical patients with disruptions to the gastrointestinal tract (30), but consensus recommendations for this practice are not established.

CANDIDIASIS OF THE CNS

Candida infects the central nervous system (CNS) as a result of hematogenously disseminated candidiasis or a complication of cerebrospinal fluid (CSF) shunt placement or other neurosurgical procedures (20, 46, 104, 138, 139, 148, 160). Meningitis is the most common manifestation and occurs in conjunction with infections of other organs in about half the cases. Infections of the brain parenchyma include multiple microabscesses and small macroabscesses and, more rarely, single large abscesses or epidural abscesses (14, 15, 34, 126). The vast majority of CNS candidiasis reported to date is caused by *C. albicans*, with other species implicated much less frequently (14, 46, 104, 148, 160).

Patients with meningitis usually present with subacute onset of symptoms like fever, headache, mental status changes, and irritability. Stiff neck, a classic finding of meningeal irritation in bacterial meningitis, is uncommon. In some patients, the signs and symptoms of *Candida* meningitis are nonspecific, which may delay diagnostic evaluation. In addition, it may be difficult to elicit or recognize findings in otherwise gravely ill patients. CSF analysis reveals leukocytosis (average count of 600 cells/mm^3), which demonstrates a lymphocyte predominance in about half the cases. Sixty percent of patients have low glucose and high protein levels, and CSF Gram stain is positive in 40%. The sensitivity of CSF culture is about 80% (104). The yield of CSF culture may be improved with large-volume samples (46). The presentation of *Candida* brain abscesses is variable but often includes headache and localizing neurologic symptoms.

The treatment recommendations for CNS candidiasis are derived from case reports and small case series. Based on this literature and data from a rabbit model of meningoencephalitis, the front-line regimen for both meningitis and parenchymal infection is liposomal amphotericin B combined with flucytosine (20, 46, 103, 148, 160). Liposomal amphotericin B is favored over other formulations because it may achieve higher concentrations in the brain (148). Flucytosine provides synergy and attains high concentrations in the brain (148). In exceptionally severe cases, intrathecal antifungals should be considered. Typically, the preceding regimens are given for several weeks while improvements in clinical and CSF findings are tracked. Thereafter, fluconazole (400 to 800 mg daily) step-down therapy is initiated (104, 138, 139). Fluconazole achieves excellent concentrations within the brain but is not recommended up-front because of its fungistatic rather than fungicidal activity (35, 46, 104, 138, 139). Experience with the echinocandins is very limited, and these agents are not recommended (76, 126). Antifungal therapy continues until all signs and symptoms, CSF abnormalities, and radiologic abnormalities have resolved. Removal and replacement of infected ventricular devices by a two-step process are recommended in conjunction with antifungal therapy (46, 139). There are no clear-cut indications for surgical drainage or resection of *Candida* brain abscesses, and these decisions must be made on a case-by-case basis in conjunction with neurosurgical consultation.

CANDIDIASIS OF THE CARDIOVASCULAR SYSTEM

Candida species are capable of infecting the heart valves (endocarditis), myocardium (myocarditis), pericardium (pericarditis), blood vessels, and pacemaker wires and other implantable cardiac devices. *Candida* endocarditis is the most common type of fungal endocarditis (28). Risk factors include underlying valvular heart disease, the presence of prosthetic valves, injection drug use, intravenous catheters, receipt of broad-spectrum antibiotics, and immunosuppression (33, 99, 101, 137). Over 50% of patients have undergone prosthetic valve surgery (33), as the foreign body represents a nidus for *Candida* to adhere and proliferate. Most cases of prosthetic valve endocarditis occur within the first 2 months of valve placement (34, 96, 101). Compared to other end-organ infections, *Candida* endocarditis is notable for the frequent involvement of non-*C. albicans* species. The aortic and mitral valves are most commonly infected.

The presentation, physical findings, and complications of *Candida* endocarditis are similar to those of bacterial endocarditis, including fevers, heart murmurs, hepatosplenomegaly, Osler's nodes and Janeway lesions, splinter hemorrhages, and urinary abnormalities (hematuria, proteinuria, pyuria, and casts). Large valvular vegetations, emboli to major blood vessels, and endophthalmitis are more common with *Candida* than bacterial endocarditis (137). Complications include valve perforation, myocarditis, congestive heart failure, mycotic aneurysms, and events resulting from major embolic events. Blood cultures are positive in 70 to 85% of cases (137, 144). In cases with negative blood cultures, diagnosis historically has depended upon culture or histopathology of surgically removed valves (137). As for bacterial endocarditis, transthoracic echocardiography and, increasingly, transesophageal echocardiography are a standard part of the evaluation, with the latter significantly more sensitive for the identification of vegetations.

The optimal treatment of *Candida* endocarditis is valvular replacement combined with prolonged antifungal therapy, an approach that has reduced mortality rates from almost 100% to less than 50% (18, 33, 47, 90, 97, 152). The front-line antifungal therapy is a lipid formulation of amphotericin B, with or without flucytosine (33, 75, 86, 97, 105, 152). Lipid formulations are preferred over amphotericin B deoxycholate to minimize toxicity with prolonged treatment. Echinocandins are an alternative (7, 24, 62, 77, 80, 94, 95) which may be employed in the face of amphotericin B toxicity. Valvular replacement is undertaken as soon as possible, rather than waiting until after a course of antifungal therapy. Antifungal therapy should be continued for at least 6 weeks after valvular replacement, with longer courses in the settings of perivalvular abscesses, residual infection after surgery, or other complications. Given the low rates of toxicity with fluconazole, long-term oral suppressive therapy with this agent is often employed (80, 119). Clinicians must be aware that the relapse rate among patients who survive *Candida* endocarditis is 30% (34, 96, 137), with cases reported up to 9 years after valve replacement (90). For patients in whom valvular replacement is not possible, lifelong suppressive therapy is necessary. While there are reports of successful antifungal therapy in the absence of valvular replacement, breakthrough infections during suppressive therapy are also recognized (1, 19, 25, 62, 72, 89, 101, 129, 157, 169).

Candida myocarditis (infection of the heart muscle) and pericarditis (infection of the tissue covering the heart) are most commonly encountered in patients who are immunosuppressed or who have undergone cardiac surgery (19, 34, 43, 88, 128). Myocarditis also complicates a significant percentage of cases of candidemia and is often recognized only at autopsy (39). The major complication of myocarditis is cardiac arrhythmia due to invasion of the conduction system (118). Therefore, clinicians should suspect the disease in patients with candidemia and arrhythmias. The treatment of *Candida* myocarditis is similar to that of endocarditis. *Candida* pericarditis is a rare disease that is diagnosed by culture or histopathology of pericardial fluid or tissue (19, 88, 128). The presentation is typically nonspecific. Front-line therapy for pericarditis consists of lipid formulations of amphotericin B combined with a pericardial window or pericardiectomy (67, 100, 128, 142). Alternative antifungal regimens include an echinocandin or fluconazole. Therapy is continued for several months, with step-down fluconazole an option among patients infected with susceptible isolates after an initial response to amphotericin B or an echinocandin.

Other *Candida* infections of the cardiovascular system have increased in frequency as the use of intravascular catheters, pacemakers, and other cardiac devices has increased. Blood vessel walls that are damaged by catheter insertion or other reasons are susceptible to infiltration by *Candida* species, resulting in infected blood clots (thrombophlebitis). Patients with *Candida* thrombophlebitis are usually febrile and may have swelling, warmth, or pain at the infected vessel. Such infections should be suspected in the setting of persistent candidemia despite seemingly appropriate antifungal therapy. Treatment consists of catheter removal and excision of the infected vein, followed by antifungal therapy for 2 weeks (115). When a central vein is infected, surgery is not usually an option, and antifungal therapy should be continued until the clot is resolved. Considerations for choice of antifungal agent are as discussed for candidemia (113). For infected pacemakers and implanted cardiac devices, removal of the pacemaker or entire device is combined with amphotericin B product or an echinocandin. If only the generator or pocket is infected, 4 weeks of therapy following removal of the device is recommended. If a wire is infected, at least 6 weeks of therapy is recommended following the wire removal. For ventricular assist devices that cannot be removed, treatment is the same as for endocarditis. After the bloodstream is sterilized, suppressive therapy with fluconazole should be used until the device is removed.

CANDIDA OSTEOMYELITIS AND SEPTIC ARTHRITIS

Candida bone (osteomyelitis) and joint (septic arthritis) infections result most commonly from hematogenous dissemination and less often from direct spread from contiguous sites (34, 45, 52, 98, 146). The most common sites of osteomyelitis in adults are the vertebrae and intervertebral discs. As mentioned above, osteomyelitis is often a late manifestation of previously unrecognized candidemia. The presentation is variable and may include localized pain, drainage, and fevers. At the time of clinically apparent bone disease, blood cultures are usually negative. Diagnosis is made by culture and/or histopathology of bone samples obtained by aspiration, biopsy, or surgical resection. Radiographic studies show changes typical of osteomyelitis by other pathogens, and laboratory studies invariably reveal elevated inflammatory markers such as sedimentation rate and C-reactive protein. The inflammatory markers are useful to monitor over time to gauge the response to therapy. The white blood cell count is elevated in some, but not all, cases. Septic arthritis that does not stem from direct hematogenous seeding of the joint is usually a result of trauma, intra-articular injections, or surgery (10). Infections involve both native and prosthetic joints, most commonly the knees. *Candida* arthritis generally presents more acutely than osteomyelitis, and signs and symptoms include fevers and joint tenderness, swelling, warmth, and redness. Diagnosis is made by cultures of joint aspirates.

In general, osteomyelitis is best treated with the combination of surgical debridement and prolonged antifungal therapy, although the role of surgery in the routine management of vertebral *Candida* osteomyelitis is controversial. In the event of osteomyelitis associated with hardware, surgical removal is necessary. The antifungal options include initial therapy with a lipid formulation of amphotericin B or an echinocandin (the most experience is with caspofungin) followed by fluconazole step-down therapy, or fluconazole as up-front therapy. In the first instance, the duration of lipid formulation amphotericin B or caspofungin is at least 2 weeks. The overall duration of antifungal therapy with both strategies is usually 6 to 12 months. In the event of infected hardware that cannot be removed, chronic suppressive antifungal therapy with fluconazole is required. For septic arthritis, joint drainage and surgical debridement are crucial. Hip infections need to be treated with open surgical drainage and debridement. Six weeks of antifungal therapy from the time of debridement is given, consisting of fluconazole or amphotericin B induction therapy followed by fluconazole. For prosthetic joint infections, the joint is removed, if possible (53, 147, 168). If an infected prosthetic joint is removed, a new prosthesis can be inserted after the infection is demonstrated to be eliminated. This will require at least 6 weeks of antifungal therapy and perhaps longer. As for osteomyelitis associated with retained hardware, septic arthritis with a retained prosthesis mandates chronic suppressive therapy with fluconazole.

CANDIDIASIS OF THE UPPER URINARY TRACT

The kidneys are among the most common target organs during bloodstream *Candida* infections, and the isolation of *Candida* in the urine (candiduria) may be one of the earliest manifestations of disseminated candidiasis (41, 58, 65, 78, 109, 122, 167). Therefore, candiduria should be recognized as a potential marker for disseminated candidiasis in high-risk patients (see risk factors for candidemia, above). The treatment of candiduria in the setting of disseminated candidiasis is as described earlier for candidemia. At the same time, it is important to recognize that the vast majority of cases of candiduria do not represent disseminated candidiasis. Rather, candiduria most commonly reflects colonization of the lower urinary tract, which is often encountered in patients with urinary catheters who are receiving broad-spectrum antibiotics (65). Less frequently, candiduria is encountered during pyelonephritis (infection of the renal pelvis), which develops as a result of ascending infection from the lower urinary tract. Similar to candidemia, ascending *Candida* pyelonephritis is treated with 2 weeks of antifungal therapy, most commonly fluconazole. Echinocandins are less desirable in the treatment of pyelonephritis, due to poor concentration in the urinary tract.

CANDIDA PNEUMONIA

Candida species are frequently isolated in cultures of sputum and other respiratory tract samples, particularly from patients on mechanical ventilation who are treated with broad-spectrum antibiotics (31). They are rarely causes of disease within the lungs, however (23, 56, 59, 120, 141, 177). Patients with respiratory tract cultures positive for *Candida* species, therefore, should not be treated unless there is definitive proof of *Candida* pneumonia or other lung disease. *Candida* pneumonia occurs as a diffuse, nodular disease as a result of hematogenous dissemination, or as a localized infection extending from the bronchus. Even more rarely, *Candida* species are implicated in necrotizing pneumonias or lung abscesses. Diagnosis of disease caused by *Candida* species requires histopathological evidence of tissue invasion or recovery of organisms from guided aspirates of lung abscesses. Given the rarity of these entities, it is not surprising that optimal therapeutic regimens are not clearly defined. Care must be individualized for each patient and consists of extended courses of antifungal therapy. The precise indications for drainage of *Candida* lung abscesses are unclear. Abscesses are often polymicrobial, and interventional approaches generally follow those for lung abscesses caused by other pathogens.

REFERENCES

1. **Aaron, L., A. Therby, J. P. Viard, R. Lahoulou, and B. Dupont.** 2003. Successful medical treatment of *Candida albicans* in mechanical prosthetic valve endocarditis. *Scand. J. Infect. Dis.* **35:**351–352.
2. **Alden, S. M., E. Frank, and L. Flancbaum.** 1989. Abdominal candidiasis in surgical patients. *Am. Surg.* **55:**45–49.
3. **Amici, G., S. Grandesso, A. Mottola, G. Virga, G. Calconi, and C. Bocci.** 1994. Fungal peritonitis in peritoneal dialysis: critical review of six cases. *Adv. Perit. Dial.* **10:** 169–173.
4. **Anaissie, E., G. P. Bodey, H. Kantarjian, C. David, K. Barnett, E. Bow, R. Defelice, N. Downs, T. File, G. Karam, et al.** 1991. Fluconazole therapy for chronic disseminated candidiasis in patients with leukemia and prior amphotericin B therapy. *Am. J. Med.* **91:**142–150.
5. **Anaissie, E. J., J. H. Rex, O. Uzun, and S. Vartivarian.** 1998. Predictors of adverse outcome in cancer patients with candidemia. *Am. J. Med.* **104:**238–245.
6. **Anaissie, E. J., S. E. Vartivarian, D. Abi-Said, O. Uzun, H. Pinczowski, D. P. Kontoyiannis, P. Khoury, K. Papadakis, A. Gardner, I. I. Raad, J. Gilbreath, and G. P. Bodey.** 1996. Fluconazole versus amphotericin B in the treatment of hematogenous candidiasis: a matched cohort study. *Am. J. Med.* **101:**170–176.
7. **Baddley, J. W., D. K. Benjamin, Jr., M. Patel, J. Miro, E. Athan, B. Barsic, E. Bouza, L. Clara, T. Elliott, Z. Kanafani, J. Klein, S. Lerakis, D. Levine, D. Spelman, E. Rubinstein, P. Tornos, A. J. Morris, P. Pappas, V. G. Fowler, Jr., V. H. Chu, and C. Cabell.** 2008. *Candida* infective endocarditis. *Eur. J. Clin. Microbiol. Infect. Dis.* **27:** 519–529.
8. **Barrie, T.** 1987. The place of elective vitrectomy in the management of patients with *Candida* endophthalmitis. *Graefes Arch. Clin. Exp. Ophthalmol.* **225:**107–113.
9. **Bayer, A. S., M. J. Blumenkrantz, J. Z. Montgomerie, J. E. Galpin, J. W. Coburn, and L. B. Guze.** 1976. *Candida* peritonitis. Report of 22 cases and review of the English literature. *Am. J. Med.* **61:**832–840.
10. **Belzunegui, J., C. Gonzalez, L. Lopez, I. Plazaola, O. Maiz, and M. Figueroa.** 1997. Osteoarticular and muscle infectious lesions in patients with the human immunodeficiency virus. *Clin. Rheumatol.* **16:**450–453.
11. **Bennett, J. E.** 2006. Echinocandins for candidemia in adults without neutropenia. *N. Engl. J. Med.* **355:** 1154–1159.
12. **Betts, R., A. Glasmacher, J. Maertens, G. Maschmeyer, J. A. Vazquez, H. Teppler, A. Taylor, R. Lupinacci, C. Sable, and N. Kartsonis.** 2006. Efficacy of caspofungin against invasive *Candida* or invasive *Aspergillus* infections in neutropenic patients. *Cancer* **106:**466–473.
13. **Blumberg, H. M., W. R. Jarvis, J. M. Soucie, J. E. Edwards, J. E. Patterson, M. A. Pfaller, M. S. Rangel-Frausto, M. G. Rinaldi, L. Saiman, R. T. Wiblin, and R. P. Wenzel.** 2001. Risk factors for candidal bloodstream infections in surgical intensive care unit patients: the NEMIS prospective multicenter study. The National Epidemiology of Mycosis Survey. *Clin. Infect. Dis.* **33:**177–186.
14. **Bonomo, R. A., M. Strauss, R. Blinkhorn, and R. A. Salata.** 1996. *Torulopsis* (*Candida*) *glabrata*: a new pathogen found in spinal epidural abscess. *Clin. Infect. Dis.* **22:** 588–589.
15. **Boogaerts, M., D. J. Winston, E. J. Bow, G. Garber, A. C. Reboli, A. P. Schwarer, N. Novitzky, A. Boehme, E. Chwetzoff, and K. De Beule.** 2001. Intravenous and oral itraconazole versus intravenous amphotericin B deoxycholate as empirical antifungal therapy for persistent fever in neutropenic patients with cancer who are receiving broad-spectrum antibacterial therapy. A randomized, controlled trial. *Ann. Intern. Med.* **135:**412–422.
16. **Brooks, R. G.** 1989. Prospective study of *Candida* endophthalmitis in hospitalized patients with candidemia. *Arch. Intern. Med.* **149:**2226–2228.
17. **Calandra, T., J. Bille, R. Schneider, F. Mosimann, and P. Francioli.** 1989. Clinical significance of *Candida* isolated from peritoneum in surgical patients. *Lancet* **ii:** 1437–1440.
18. **Calderone, R. A., and W. M. Scheld.** 1987. Role of fibronectin in the pathogenesis of candidal infections. *Rev. Infect. Dis.* **9**(Suppl. 4)**:**S400–S403.
19. **Canver, C. C., A. K. Patel, P. Kosolcharoen, and M. C. Voytovich.** 1998. Fungal purulent constrictive pericarditis in a heart transplant patient. *Ann. Thorac. Surg.* **65:**1792–1794.
20. **Casado, J. L., C. Quereda, J. Oliva, E. Navas, A. Moreno, V. Pintado, J. Cobo, and I. Corral.** 1997. Candidal meningitis in HIV-infected patients: analysis of 14 cases. *Clin. Infect. Dis.* **25:**673–676.
21. **Castaldo, P., R. J. Stratta, R. P. Wood, R. S. Markin, K. D. Patil, M. S. Shaefer, A. N. Langnas, E. C. Reed, S. J. Li, T. J. Pillen, et al.** 1991. Clinical spectrum of fungal infections after orthotopic liver transplantation. *Arch. Surg.* **126:**149–156.
22. **Chan, T. M., C. Y. Chan, S. W. Cheng, W. K. Lo, C. Y. Lo, and I. K. Cheng.** 1994. Treatment of fungal peritonitis complicating continuous ambulatory peritoneal dialysis with oral fluconazole: a series of 21 patients. *Nephrol. Dial. Transplant.* **9:**539–542.
23. **Connolly, J. E., Jr., H. P. McAdams, J. J. Erasmus, and M. L. Rosado-de-Christenson.** 1999. Opportunistic fungal pneumonia. *J. Thorac. Imaging* **14:**51–62.
24. **Cornely, O. A., M. Lasso, R. Betts, N. Klimko, J. Vazquez, G. Dobb, J. Velez, A. Williams-Diaz, J. Lipka, A. Taylor, C. Sable, and N. Kartsonis.** 2007. Caspofungin for the treatment of less common forms of invasive candidiasis. *J. Antimicrob. Chemother.* **60:**363–369.
25. **Czwerwiec, F. S., M. S. Bilsker, M. L. Kamerman, and A. L. Bisno.** 1993. Long-term survival after fluconazole therapy of candidal prosthetic valve endocarditis. *Am. J. Med.* **94:**545–546.

26. **Diebel, L. N., A. M. Raafat, S. A. Dulchavsky, and W. J. Brown.** 1996. Gallbladder and biliary tract candidiasis. *Surgery* **120:**760–764; discussion, 764–765.
27. **Donahue, S. P., E. Hein, and R. B. Sinatra.** 2003. Ocular involvement in children with candidemia. *Am. J. Ophthalmol.* **135:**886–887.
28. **Edwards, J. E.** 2005. *Candida* species, p. 2938–2957. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), *Principles and Practice of Infectious Diseases*, 6th ed., vol. 2. Elsevier, Philadelphia, PA.
29. **Edwards, J. E., Jr.** 1992. Should all patients with candidemia be treated with antifungal agents? *Clin. Infect. Dis.* **15:**422–423.
30. **Eggimann, P., P. Francioli, J. Bille, R. Schneider, M. M. Wu, G. Chapuis, R. Chiolero, A. Pannatier, J. Schilling, S. Geroulanos, M. P. Glauser, and T. Calandra.** 1999. Fluconazole prophylaxis prevents intra-abdominal candidiasis in high-risk surgical patients. *Crit. Care Med.* **27:** 1066–1072.
31. **el-Ebiary, M., A. Torres, N. Fabregas, J. P. de la Bellacasa, J. Gonzalez, J. Ramirez, D. del Bano, C. Hernandez, and M. T. Jimenez de Anta.** 1997. Significance of the isolation of *Candida* species from respiratory samples in critically ill, non-neutropenic patients. An immediate postmortem histologic study. *Am. J. Respir. Crit. Care Med.* **156:** 583–590.
32. **Ellepola, A. N., and C. J. Morrison.** 2005. Laboratory diagnosis of invasive candidiasis. *J. Microbiol.* **43**(Spec. No.)**:** 65–84.
33. **Ellis, M. E., H. Al-Abdely, A. Sandridge, W. Greer, and W. Ventura.** 2001. Fungal endocarditis: evidence in the world literature, 1965–1995. *Clin. Infect. Dis.* **32:** 50–62.
34. **EORTC International Antimicrobial Therapy Cooperative Group.** 1989. Empiric antifungal therapy in febrile granulocytopenic patients. *Am. J. Med.* **86:**668–672.
35. **Epelbaum, S., C. Laurent, G. Morin, P. Berquin, and C. Piussan.** 1993. Failure of fluconazole treatment in *Candida* meningitis. *J. Pediatr.* **123:**168–169.
36. **Espinel-Ingroff, A., F. Barchiesi, M. Cuenca-Estrella, M. A. Pfaller, M. Rinaldi, J. L. Rodriguez-Tudela, and P. E. Verweij.** 2005. International and multicenter comparison of EUCAST and CLSI M27-A2 broth microdilution methods for testing susceptibilities of *Candida* spp. to fluconazole, itraconazole, posaconazole, and voriconazole. *J. Clin. Microbiol.* **43:**3884–3889.
37. **Feman, S. S., J. C. Nichols, S. M. Chung, and T. A. Theobald.** 2002. Endophthalmitis in patients with disseminated fungal disease. *Trans. Am. Ophthalmol. Soc.* **100:**67–70; discussion, 70–71.
38. **Fidel, P. L., Jr.** 2006. *Candida*-host interactions in HIV disease: implications for oropharyngeal candidiasis. *Adv. Dent. Res.* **23:**45–49.
39. **Filler, S. G., and B. J. Kullberg.** 2002. Deep-seated candidal infections, p. 341–348. *In* R. Calderone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
40. **Filler, S. G., and B. J. Kullberg.** 2002. Candidemia, p. 327–340. *In* R. Calderone (ed.), Candida *and Candidiasis*. ASM Press, Washington, DC.
41. **Fisher, J. F., C. L. Newman, and J. D. Sobel.** 1995. Yeast in the urine: solutions for a budding problem. *Clin. Infect. Dis.* **20:**183–189.
42. **Foust, R. T.** 1996. Infection of a pancreatic pseudocyst due to *Candida albicans*. *South. Med. J.* **89:**1104–1107.
43. **Franklin, W. G., A. B. Simon, and T. M. Sodeman.** 1976. *Candida* myocarditis without valvulitis. *Am. J. Cardiol.* **38:**924–928.
44. **Garey, K. W., M. Rege, M. P. Pai, D. E. Mingo, K. J. Suda, R. S. Turpin, and D. T. Bearden.** 2006. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. *Clin. Infect. Dis.* **43:**25–31.
45. **Gathe, J. C., Jr., R. L. Harris, B. Garland, M. W. Bradshaw, and T. W. Williams, Jr.** 1987. *Candida* osteomyelitis. Report of five cases and review of the literature. *Am. J. Med.* **82:**927–937.
46. **Geers, T. A., and S. M. Gordon.** 1999. Clinical significance of *Candida* species isolated from cerebrospinal fluid following neurosurgery. *Clin. Infect. Dis.* **28:**1139–1147.
47. **Gilbert, H. M., E. D. Peters, S. J. Lang, and B. J. Hartman.** 1996. Successful treatment of fungal prosthetic valve endocarditis: case report and review. *Clin. Infect. Dis.* **22:** 348–354.
48. **Gokhale, P. C., R. J. Barapatre, S. H. Advani, N. A. Kshirsagar, and S. K. Pandya.** 1993. Successful treatment of disseminated candidiasis resistant to amphotericin B by liposomal amphotericin B: a case report. *J. Cancer Res. Clin. Oncol.* **119:**569–571.
49. **Goldie, S. J., L. Kiernan-Tridle, C. Torres, N. Gorban-Brennan, D. Dunne, A. S. Kliger, and F. O. Finkelstein.** 1996. Fungal peritonitis in a large chronic peritoneal dialysis population: a report of 55 episodes. *Am. J. Kidney Dis.* **28:**86–91.
50. **Goodrich, J. M., E. C. Reed, M. Mori, L. D. Fisher, S. Skerrett, P. S. Dandliker, B. Klis, G. W. Counts, and J. D. Meyers.** 1991. Clinical features and analysis of risk factors for invasive candidal infection after marrow transplantation. *J. Infect. Dis.* **164:**731–740.
51. **Gudlaugsson, O., S. Gillespie, K. Lee, J. Vande Berg, J. Hu, S. Messer, L. Herwaldt, M. Pfaller, and D. Diekema.** 2003. Attributable mortality of nosocomial candidemia, revisited. *Clin. Infect. Dis.* **37:**1172–1177.
52. **Hansen, B. L., and K. Andersen.** 1995. Fungal arthritis. A review. *Scand. J. Rheumatol.* **24:**248–250.
53. **Harris, M. C., G. R. Pereira, M. D. Myers, A. J. Cardin, B. Razdan, J. Pleasure, and L. M. Bell.** 2000. Candidal arthritis in infants previously treated for systemic candidiasis during the newborn period: report of three cases. *Pediatr. Emerg. Care* **16:**249–251.
54. **Henderson, D. K., L. J. Hockey, L. J. Vukalcic, and J. E. Edwards, Jr.** 1980. Effect of immunosuppression on the development of experimental hematogenous *Candida* endophthalmitis. *Infect. Immun.* **27:**628–631.
55. **Hesse, U. J., D. E. Sutherland, R. L. Simmons, and J. S. Najarian.** 1986. Intra-abdominal infections in pancreas transplant recipients. *Ann. Surg.* **203:**153–162.
56. **Heurlin, N., S. E. Bergstrom, J. Winiarski, O. Ringden, P. Ljungman, B. Lonnqvist, and J. Andersson.** 1996. Fungal pneumonia: the predominant lung infection causing death in children undergoing bone marrow transplantation. *Acta Paediatr.* **85:**168–172.
57. **Hiatt, J. R., M. R. Kobayashi, J. E. Doty, and K. P. Ramming.** 1991. Acalculous *Candida* cholecystitis: a complication of critical surgical illness. *Am. Surg.* **57:**825–829.
58. **Hitchcock, R. J., A. Pallett, M. A. Hall, and P. S. Malone.** 1995. Urinary tract candidiasis in neonates and infants. *Br. J. Urol.* **76:**252–256.
59. **Horn, D. L., J. A. Fishman, W. J. Steinbach, E. J. Anaissie, K. A. Marr, A. J. Olyaei, M. A. Pfaller, M. A. Weiss, K. M. Webster, and D. Neofytos.** 2007. Presentation of the PATH Alliance registry for prospective data collection and analysis of the epidemiology, therapy, and outcomes of invasive fungal infections. *Diagn. Microbiol. Infect. Dis.* **59:**407–414.
60. **Horn, R., B. Wong, T. E. Kiehn, and D. Armstrong.** 1985. Fungemia in a cancer hospital: changing frequency, earlier onset, and results of therapy. *Rev. Infect. Dis.* **7:** 646–655.

61. **Jalan, R., H. L. Jones, and R. J. Walker.** 1994. Multiple pancreatic abscesses due to *Candida albicans* following ERCP. *Scott. Med. J.* **39:**17–18.
62. **Jimenez-Exposito, M. J., G. Torres, A. Baraldes, N. Benito, F. Marco, J. C. Pare, A. Moreno, X. Claramonte, C. A. Mestres, M. Almela, C. Garcia de la Maria, N. Perez, W. A. Schell, G. R. Corey, J. Perfect, M. T. Jimenez de Anta, J. M. Gatell, and J. M. Miro.** 2004. Native valve endocarditis due to *Candida glabrata* treated without valvular replacement: a potential role for caspofungin in the induction and maintenance treatment. *Clin. Infect. Dis.* **39:**e70–e73.
63. **Karlowicz, M. G., L. N. Hashimoto, R. E. Kelly, Jr., and E. S. Buescher.** 2000. Should central venous catheters be removed as soon as candidemia is detected in neonates? *Pediatrics* **106:**E63.
64. **Kauffman, C. A., S. F. Bradley, S. C. Ross, and D. R. Weber.** 1991. Hepatosplenic candidiasis: successful treatment with fluconazole. *Am. J. Med.* **91:**137–141.
65. **Kauffman, C. A., J. A. Vazquez, J. D. Sobel, H. A. Gallis, D. S. McKinsey, A. W. Karchmer, A. M. Sugar, P. K. Sharkey, G. J. Wise, R. Mangi, A. Mosher, J. Y. Lee, and W. E. Dismukes.** 2000. Prospective multicenter surveillance study of funguria in hospitalized patients. The National Institute for Allergy and Infectious Diseases (NIAID) Mycoses Study Group. *Clin. Infect. Dis.* **30:**14–18.
66. **Komshian, S. V., A. K. Uwaydah, J. D. Sobel, and L. R. Crane.** 1989. Fungemia caused by *Candida* species and *Torulopsis glabrata* in the hospitalized patient: frequency, characteristics, and evaluation of factors influencing outcome. *Rev. Infect. Dis.* **11:**379–390.
67. **Kraus, W. E., P. N. Valenstein, and G. R. Corey.** 1988. Purulent pericarditis caused by *Candida*: report of three cases and identification of high-risk populations as an aid to early diagnosis. *Rev. Infect. Dis.* **10:**34–41.
68. **Kullberg, B. J., J. D. Sobel, M. Ruhnke, P. G. Pappas, C. Viscoli, J. H. Rex, J. D. Cleary, E. Rubinstein, L. W. Church, J. M. Brown, H. T. Schlamm, I. T. Oborska, F. Hilton, and M. R. Hodges.** 2005. Voriconazole versus a regimen of amphotericin B followed by fluconazole for candidaemia in non-neutropenic patients: a randomised non-inferiority trial. *Lancet* **366:**1435–1442.
69. **Kuse, E. R., P. Chetchotisakd, C. A. da Cunha, M. Ruhnke, C. Barrios, D. Raghunadharao, J. S. Sekhon, A. Freire, V. Ramasubramanian, I. Demeyer, M. Nucci, A. Leelarasamee, F. Jacobs, J. Decruyenaere, D. Pittet, A. J. Ullmann, L. Ostrosky-Zeichner, O. Lortholary, S. Koblinger, H. Diekmann-Berndt, and O. A. Cornely.** 2007. Micafungin versus liposomal amphotericin B for candidaemia and invasive candidosis: a phase III randomised double-blind trial. *Lancet* **369:**1519–1527.
70. **Lecciones, J. A., J. W. Lee, E. E. Navarro, F. G. Witebsky, D. Marshall, S. M. Steinberg, P. A. Pizzo, and T. J. Walsh.** 1992. Vascular catheter-associated fungemia in patients with cancer: analysis of 155 episodes. *Clin. Infect. Dis.* **14:**875–883.
71. **Legrand, F., M. Lecuit, B. Dupont, E. Bellaton, M. Huerre, P. S. Rohrlich, and O. Lortholary.** 2008. Adjuvant corticosteroid therapy for chronic disseminated candidiasis. *Clin. Infect. Dis.* **46:**696–702.
72. **Lejko-Zupanc, T., and M. Kozelj.** 1997. A case of recurrent *Candida parapsilosis* prosthetic valve endocarditis: cure by medical treatment alone. *J. Infect.* **35:**81–82.
73. **Leleu, G., P. Aegerter, and B. Guidet.** 2002. Systemic candidiasis in intensive care units: a multicenter, matched-cohort study. *J. Crit. Care* **17:**168–175.
74. **Leon, C., S. Ruiz-Santana, P. Saavedra, B. Almirante, J. Nolla-Salas, F. Alvarez-Lerma, J. Garnacho-Montero, and M. A. Leon.** 2006. A bedside scoring system ("*Candida* score") for early antifungal treatment in nonneutropenic critically ill patients with *Candida* colonization. *Crit. Care Med.* **34:**730–737.
75. **Levy, I., I. Shalit, E. Birk, L. Sirota, S. Ashkenazi, B. German, and N. Linder.** 2006. *Candida* endocarditis in neonates: report of five cases and review of the literature. *Mycoses* **49:**43–48.
76. **Liu, K. H., C. J. Wu, C. H. Chou, H. C. Lee, N. Y. Lee, S. T. Hung, and W. C. Ko.** 2004. Refractory candidal meningitis in an immunocompromised patient cured by caspofungin. *J. Clin. Microbiol.* **42:**5950–5953.
77. **Lopez-Ciudad, V., M. J. Castro-Orjales, C. Leon, C. Sanz-Rodriguez, M. J. de la Torre-Fernandez, M. A. Perez de Juan-Romero, M. D. Collell-Llach, and M. D. Diaz-Lopez.** 2006. Successful treatment of *Candida parapsilosis* mural endocarditis with combined caspofungin and voriconazole. *BMC Infect. Dis.* **6:**73.
78. **Lundstrom, T., and J. Sobel.** 2001. Nosocomial candiduria: a review. *Clin. Infect. Dis.* **32:**1602–1607.
79. **Luzzati, R., G. Amalfitano, L. Lazzarini, F. Soldani, S. Bellino, M. Solbiati, M. C. Danzi, S. Vento, G. Todeschini, C. Vivenza, and E. Concia.** 2000. Nosocomial candidemia in non-neutropenic patients at an Italian tertiary care hospital. *Eur. J. Clin. Microbiol. Infect. Dis.* **19:**602–607.
80. **Lye, D. C., A. Hughes, D. O'Brien, and E. Athan.** 2005. *Candida glabrata* prosthetic valve endocarditis treated successfully with fluconazole plus caspofungin without surgery: a case report and literature review. *Eur. J. Clin. Microbiol. Infect. Dis.* **24:**753–755.
81. **Maksymiuk, A. W., S. Thongprasert, R. Hopfer, M. Luna, V. Fainstein, and G. P. Bodey.** 1984. Systemic candidiasis in cancer patients. *Am. J. Med.* **77:**20–27.
82. **Malik, I. A., I. Moid, Z. Aziz, S. Khan, and M. Suleman.** 1998. A randomized comparison of fluconazole with amphotericin B as empiric anti-fungal agents in cancer patients with prolonged fever and neutropenia. *Am. J. Med.* **105:**478–483.
83. **Marchetti, O., J. Bille, U. Fluckiger, P. Eggimann, C. Ruef, J. Garbino, T. Calandra, M. P. Glauser, M. G. Tauber, and D. Pittet.** 2004. Epidemiology of candidemia in Swiss tertiary care hospitals: secular trends, 1991–2000. *Clin. Infect. Dis.* **38:**311–320.
84. **Martino, P., C. Girmenia, A. Micozzi, F. De Bernardis, M. Boccanera, and A. Cassone.** 1994. Prospective study of *Candida* colonization, use of empiric amphotericin B and development of invasive mycosis in neutropenic patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **13:**797–804.
85. **Martino, R., M. Subira, A. Sureda, and J. Sierra.** 1999. Amphotericin B lipid complex at 3 mg/kg/day for treatment of invasive fungal infections in adults with haematological malignancies. *J. Antimicrob. Chemother.* **44:**569–572.
86. **Mayayo, E., J. Moralejo, J. Camps, and J. Guarro.** 1996. Fungal endocarditis in premature infants: case report and review. *Clin. Infect. Dis.* **22:**366–368.
87. **McDonald, H. R., S. De Bustros, and J. O. Sipperley.** 1990. Vitrectomy for epiretinal membrane with *Candida* chorioretinitis. *Ophthalmology* **97:**466–469.
88. **McNamee, C. J., S. Wang, and D. Modry.** 1998. Purulent pericarditis secondary to *Candida parapsilosis* and *Peptostreptococcus* species. *Can. J. Cardiol.* **14:**85–86.
89. **Melamed, R., E. Leibovitz, O. Abramson, A. Levitas, N. Zucker, and R. Gorodisher.** 2000. Successful nonsurgical treatment of *Candida tropicalis* endocarditis with liposomal amphotericin-B (AmBisome). *Scand. J. Infect. Dis.* **32:**86–89.
90. **Melgar, G. R., R. M. Nasser, S. M. Gordon, B. W. Lytle, T. F. Keys, and D. L. Longworth.** 1997. Fungal prosthetic valve endocarditis in 16 patients. An 11-year experience in a tertiary care hospital. *Medicine* (Baltimore) **76:**94–103.

91. **Montenegro, J., R. Aguirre, O. Gonzalez, I. Martinez, and R. Saracho.** 1995. Fluconazole treatment of *Candida* peritonitis with delayed removal of the peritoneal dialysis catheter. *Clin. Nephrol.* **44:**60–63.
92. **Mora-Duarte, J., R. Betts, C. Rotstein, A. L. Colombo, L. Thompson-Moya, J. Smietana, R. Lupinacci, C. Sable, N. Kartsonis, and J. Perfect.** 2002. Comparison of caspofungin and amphotericin B for invasive candidiasis. *N. Engl. J. Med.* **347:**2020–2029.
93. **Morrell, M., V. J. Fraser, and M. H. Kollef.** 2005. Delaying the empiric treatment of *Candida* bloodstream infection until positive blood culture results are obtained: a potential risk factor for hospital mortality. *Antimicrob. Agents Chemother.* **49:**3640–3645.
94. **Moudgal, V., T. Little, D. Boikov, and J. A. Vazquez.** 2005. Multiechinocandin- and multiazole-resistant *Candida parapsilosis* isolates serially obtained during therapy for prosthetic valve endocarditis. *Antimicrob. Agents Chemother.* **49:**767–769.
95. **Mrowczynski, W., and M. Wojtalik.** 2004. Caspofungin for *Candida* endocarditis. *Pediatr. Infect. Dis. J.* **23:**376.
96. **Muehrcke, D. D.** 1995. Fungal prosthetic valve endocarditis. *Semin. Thorac. Cardiovasc. Surg.* **7:**20–24.
97. **Muehrcke, D. D., B. W. Lytle, and D. M. Cosgrove III.** 1995. Surgical and long-term antifungal therapy for fungal prosthetic valve endocarditis. *Ann. Thorac. Surg.* **60:** 538–543.
98. **Murphy, O., J. Gray, J. Wagget, and S. J. Pedler.** 1997. Candida arthritis complicating long term total parenteral nutrition. *Pediatr. Infect. Dis. J.* **16:**329.
99. **Nasser, R. M., G. R. Melgar, D. L. Longworth, and S. M. Gordon.** 1997. Incidence and risk of developing fungal prosthetic valve endocarditis after nosocomial candidemia. *Am. J. Med.* **103:**25–32.
100. **Neughebauer, B., V. Alvarez, T. Harb, and M. Keefer.** 2002. Constrictive pericarditis caused by *Candida glabrata* in an immunocompetent patient: case report and review of literature. *Scand. J. Infect. Dis.* **34:**615–619.
101. **Nguyen, M. H., M. L. Nguyen, V. L. Yu, D. McMahon, T. F. Keys, and M. Amidi.** 1996. *Candida* prosthetic valve endocarditis: prospective study of six cases and review of the literature. *Clin. Infect. Dis.* **22:**262–267.
102. **Nguyen, M. H., J. E. Peacock, Jr., A. J. Morris, D. C. Tanner, M. L. Nguyen, D. R. Snydman, M. M. Wagener, M. G. Rinaldi, and V. L. Yu.** 1996. The changing face of candidemia: emergence of non-*Candida albicans* species and antifungal resistance. *Am. J. Med.* **100:**617–623.
103. **Nguyen, M. H., J. E. Peacock, Jr., D. C. Tanner, A. J. Morris, M. L. Nguyen, D. R. Snydman, M. M. Wagener, and V. L. Yu.** 1995. Therapeutic approaches in patients with candidemia. Evaluation in a multicenter, prospective, observational study. *Arch. Intern. Med.* **155:** 2429–2435.
104. **Nguyen, M. H., and V. L. Yu.** 1995. Meningitis caused by *Candida* species: an emerging problem in neurosurgical patients. *Clin. Infect. Dis.* **21:**323–327.
105. **Noyola, D. E., M. Fernandez, E. H. Moylett, and C. J. Baker.** 2001. Ophthalmologic, visceral, and cardiac involvement in neonates with candidemia. *Clin. Infect. Dis.* **32:**1018–1023.
106. **Nucci, M., E. Anaissie, R. F. Betts, B. F. Dupont, C. Wu, D. N. Buell, L. Kovanda, and O. Lortholary.** 2010. Early removal of central venous catheter in patients with candidemia does not improve outcome: analysis of 842 patients from 2 randomized clinical trials. *Clin. Infect. Dis.* **51:**295–303.
107. **Obayashi, T., K. Negishi, T. Suzuki, and N. Funata.** 2008. Reappraisal of the serum (1→3)-beta-D-glucan assay for the diagnosis of invasive fungal infections—a study based on autopsy cases from 6 years. *Clin. Infect. Dis.* **46:**1864–1870.
108. **Odabasi, Z., G. Mattiuzzi, E. Estey, H. Kantarjian, F. Saeki, R. J. Ridge, P. A. Ketchum, M. A. Finkelman, J. H. Rex, and L. Ostrosky-Zeichner.** 2004. Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. *Clin. Infect. Dis.* **39:**199–205.
109. **Oravcova, E., J. Lacka, L. Drgona, M. Studena, L. Sevcikova, S. Spanik, J. Svec, E. Kukuckova, E. Grey, J. Silva, and V. Krcmery.** 1996. Funguria in cancer patients: analysis of risk factors, clinical presentation and outcome in 50 patients. *Infection* **24:**319–323.
110. **Ostrosky-Zeichner, L., B. D. Alexander, D. H. Kett, J. Vazquez, P. G. Pappas, F. Saeki, P. A. Ketchum, J. Wingard, R. Schiff, H. Tamura, M. A. Finkelman, and J. H. Rex.** 2005. Multicenter clinical evaluation of the (1→3) beta-D-glucan assay as an aid to diagnosis of fungal infections in humans. *Clin. Infect. Dis.* **41:**654–659.
111. **Ostrosky-Zeichner, L., A. M. Oude Lashof, B. J. Kullberg, and J. H. Rex.** 2003. Voriconazole salvage treatment of invasive candidiasis. *Eur. J. Clin. Microbiol. Infect. Dis.* **22:**651–655.
112. **Ostrosky-Zeichner, L., C. Sable, J. Sobel, B. D. Alexander, G. Donowitz, V. Kan, C. A. Kauffman, D. Kett, R. A. Larsen, V. Morrison, M. Nucci, P. G. Pappas, M. E. Bradley, S. Major, L. Zimmer, D. Wallace, W. E. Dismukes, and J. H. Rex.** 2007. Multicenter retrospective development and validation of a clinical prediction rule for nosocomial invasive candidiasis in the intensive care setting. *Eur. J. Clin. Microbiol. Infect. Dis.* **26:**271–276.
113. **Pan, S. C., S. M. Hsieh, S. C. Chang, H. T. Lee, and Y. C. Chen.** 2005. Septic *Candida krusei* thrombophlebitis of inferior vena cava with persistent fungemia successfully treated by new antifungal agents. *Med. Mycol.* **43:** 731–734.
114. **Paphitou, N. I., L. Ostrosky-Zeichner, and J. H. Rex.** 2005. Rules for identifying patients at increased risk for candidal infections in the surgical intensive care unit: approach to developing practical criteria for systematic use in antifungal prophylaxis trials. *Med. Mycol.* **43:** 235–243.
115. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, and J. D. Sobel.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.
116. **Pappas, P. G., J. H. Rex, J. Lee, R. J. Hamill, R. A. Larsen, W. Powderly, C. A. Kauffman, N. Hyslop, J. E. Mangino, S. Chapman, H. W. Horowitz, J. E. Edwards, and W. E. Dismukes.** 2003. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin. Infect. Dis.* **37:**634–643.
117. **Pappas, P. G., C. M. Rotstein, R. F. Betts, M. Nucci, D. Talwar, J. J. De Waele, J. A. Vazquez, B. F. Dupont, D. L. Horn, L. Ostrosky-Zeichner, A. C. Reboli, B. Suh, R. Digumarti, C. Wu, L. L. Kovanda, L. J. Arnold, and D. N. Buell.** 2007. Micafungin versus caspofungin for treatment of candidemia and other forms of invasive candidiasis. *Clin. Infect. Dis.* **45:**883–893.
118. **Parker, J. C., Jr.** 1980. The potentially lethal problem of cardiac candidosis. *Am. J. Clin. Pathol.* **73:**356–361.
119. **Penk, A., and L. Pittrow.** 1999. Role of fluconazole in the long-term suppressive therapy of fungal infections in

patients with artificial implants. *Mycoses* **42**(Suppl. 2)**:** 91–96.
120. **Petrocheilou-Paschou, V., K. Georgilis, D. Kontoyannis, J. Nanas, H. Prifti, H. Costopoulos, and S. Stamatelopoulos.** 2002. Pneumonia due to *Candida krusei*. *Clin. Microbiol. Infect.* **8:**806–809.
121. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
122. **Phillips, J. R., and M. G. Karlowicz.** 1997. Prevalence of *Candida* species in hospital-acquired urinary tract infections in a neonatal intensive care unit. *Pediatr. Infect. Dis. J.* **16:**190–194.
123. **Piarroux, R., F. Grenouillet, P. Balvay, V. Tran, G. Blasco, L. Millon, and A. Boillot.** 2004. Assessment of preemptive treatment to prevent severe candidiasis in critically ill surgical patients. *Crit. Care Med.* **32:**2443–2449.
124. **Pittet, D., M. Monod, P. M. Suter, E. Frenk, and R. Auckenthaler.** 1994. *Candida* colonization and subsequent infections in critically ill surgical patients. *Ann. Surg.* **220:**751–758.
125. **Pizzo, P. A., K. J. Robichaud, F. A. Gill, and F. G. Witebsky.** 1982. Empiric antibiotic and antifungal therapy for cancer patients with prolonged fever and granulocytopenia. *Am. J. Med.* **72:**101–111.
126. **Prabhu, R. M., and R. Orenstein.** 2004. Failure of caspofungin to treat brain abscesses secondary to *Candida albicans* prosthetic valve endocarditis. *Clin. Infect. Dis.* **39:**1253–1254.
127. **Puzniak, L., S. Teutsch, W. Powderly, and L. Polish.** 2004. Has the epidemiology of nosocomial candidemia changed? *Infect. Control Hosp. Epidemiol.* **25:**628–633.
128. **Rabinovici, R., D. Szewczyk, P. Ovadia, J. R. Greenspan, and J. J. Sivalingam.** 1997. *Candida* pericarditis: clinical profile and treatment. *Ann. Thorac. Surg.* **63:**1200–1204.
129. **Rajendram, R., N. J. Alp, A. R. Mitchell, I. C. Bowler, and J. C. Forfar.** 2005. *Candida* prosthetic valve endocarditis cured by caspofungin therapy without valve replacement. *Clin. Infect. Dis.* **40:**e72–e74.
130. **Reboli, A. C., C. Rotstein, P. G. Pappas, S. W. Chapman, D. H. Kett, D. Kumar, R. Betts, M. Wible, B. P. Goldstein, J. Schranz, D. S. Krause, and T. J. Walsh.** 2007. Anidulafungin versus fluconazole for invasive candidiasis. *N. Engl. J. Med.* **356:**2472–2482.
131. **Rex, J. H., J. E. Bennett, A. M. Sugar, P. G. Pappas, J. Serody, J. E. Edwards, and R. G. Washburn.** 1995. Intravascular catheter exchange and duration of candidemia. NIAID Mycoses Study Group and the Candidemia Study Group. *Clin. Infect. Dis.* **21:**994–996.
132. **Rex, J. H., J. E. Bennett, A. M. Sugar, P. G. Pappas, C. M. van der Horst, J. E. Edwards, R. G. Washburn, W. M. Scheld, A. W. Karchmer, A. P. Dine, et al.** 1994. A randomized trial comparing fluconazole with amphotericin B for the treatment of candidemia in patients without neutropenia. Candidemia Study Group and the National Institute. *N. Engl. J. Med.* **331:**1325–1330.
133. **Rex, J. H., P. G. Pappas, A. W. Karchmer, J. Sobel, J. E. Edwards, S. Hadley, C. Brass, J. A. Vazquez, S. W. Chapman, H. W. Horowitz, M. Zervos, D. McKinsey, J. Lee, T. Babinchak, R. W. Bradsher, J. D. Cleary, D. M. Cohen, L. Danziger, M. Goldman, J. Goodman, E. Hilton, N. E. Hyslop, D. H. Kett, J. Lutz, R. H. Rubin, W. M. Scheld, M. Schuster, B. Simmons, D. K. Stein, R. G. Washburn, L. Mautner, T. C. Chu, H. Panzer, R. B. Rosenstein, and J. Booth.** 2003. A randomized and blinded multicenter trial of high-dose fluconazole plus placebo versus fluconazole plus amphotericin B as therapy for candidemia and its consequences in nonneutropenic subjects. *Clin. Infect. Dis.* **36:**1221–1228.
134. **Rex, J. H., T. J. Walsh, J. D. Sobel, S. G. Filler, P. G. Pappas, W. E. Dismukes, and J. E. Edwards.** 2000. Practice guidelines for the treatment of candidiasis. Infectious Diseases Society of America. *Clin. Infect. Dis.* **30:** 662–678.
135. **Rodriguez-Adrian, L. J., R. T. King, L. G. Tamayo-Derat, J. W. Miller, C. A. Garcia, and J. H. Rex.** 2003. Retinal lesions as clues to disseminated bacterial and candidal infections: frequency, natural history, and etiology. *Medicine* (Baltimore) **82:**187–202.
136. **Rodriguez-Perez, J. C.** 1987. Fungal peritonitis in CAPD—which treatment is best? *Contrib. Nephrol.* **57:** 114–121.
137. **Rubinstein, E., and R. Lang.** 1995. Fungal endocarditis. *Eur. Heart J.* **16**(Suppl. B)**:**84–89.
138. **Sanchez-Portocarrero, J., P. Martin-Rabadan, C. J. Saldana, and E. Perez-Cecilia.** 1994. *Candida* cerebrospinal fluid shunt infection. Report of two new cases and review of the literature. *Diagn. Microbiol. Infect. Dis.* **20:** 33–40.
139. **Sanchez-Portocarrero, J., E. Perez-Cecilia, O. Corral, J. Romero-Vivas, and J. J. Picazo.** 2000. The central nervous system and infection by *Candida* species. *Diagn. Microbiol. Infect. Dis.* **37:**169–179.
140. **Sandven, P., H. Qvist, E. Skovlund, and K. E. Giercksky.** 2002. Significance of *Candida* recovered from intraoperative specimens in patients with intra-abdominal perforations. *Crit. Care Med.* **30:**541–547.
141. **Saubolle, M. A.** 2000. Fungal pneumonias. *Semin. Respir. Infect.* **15:**162–177.
142. **Schrank, J. H., Jr., and D. P. Dooley.** 1995. Purulent pericarditis caused by *Candida* species: case report and review. *Clin. Infect. Dis.* **21:**182–187.
143. **Schuster, M. G., J. E. Edwards, Jr., J. D. Sobel, R. O. Darouiche, A. W. Karchmer, S. Hadley, G. Slotman, H. Panzer, P. Biswas, and J. H. Rex.** 2008. Empirical fluconazole versus placebo for intensive care unit patients: a randomized trial. *Ann. Intern. Med.* **149:**83–90.
144. **Seelig, M. S., C. P. Speth, P. J. Kozinn, C. L. Taschdjian, E. F. Toni, and P. Goldberg.** 1974. Patterns of *Candida* endocarditis following cardiac surgery: importance of early diagnosis and therapy (an analysis of 91 cases). *Prog. Cardiovasc. Dis.* **17:**125–160.
145. **Shan, Y. S., E. D. Sy, S. T. Wang, J. C. Lee, and P. W. Lin.** 2006. Early presumptive therapy with fluconazole for occult *Candida* infection after gastrointestinal surgery. *World J. Surg.* **30:**119–126.
146. **Silveira, L. H., M. L. Cuellar, G. Citera, G. E. Cabrera, E. Scopelitis, and L. R. Espinoza.** 1993. *Candida* arthritis. *Rheum. Dis. Clin. N. Am.* **19:**427–437.
147. **Sim, J. P., B. C. Kho, H. S. Liu, R. Yung, and J. C. Chan.** 2005. *Candida tropicalis* arthritis of the knee in a patient with acute lymphoblastic leukaemia: successful treatment with caspofungin. *Hong Kong Med. J.* **11:**120–123.
148. **Smego, R. A., Jr., J. R. Perfect, and D. T. Durack.** 1984. Combined therapy with amphotericin B and 5-fluorocytosine for *Candida* meningitis. *Rev. Infect. Dis.* **6:**791–801.
149. **Sobel, J. D.** 1988. Pathogenesis and epidemiology of vulvovaginal candidiasis. *Ann. N. Y. Acad. Sci.* **544:** 547–557.
150. **Solomkin, J. S., A. B. Flohr, P. G. Quie, and R. L. Simmons.** 1980. The role of *Candida* in intraperitoneal infections. *Surgery* **88:**524–530.
151. **Sora, F., P. Chiusolo, N. Piccirillo, L. Pagano, L. Laurenti, G. Farina, S. Sica, and G. Leone.** 2002. Successful treatment with caspofungin of hepatosplenic candidiasis resistant to liposomal amphotericin B. *Clin. Infect. Dis.* **35:**1135–1136.

152. **Steinbach, W. J., J. R. Perfect, C. H. Cabell, V. G. Fowler, G. R. Corey, J. S. Li, A. K. Zaas, and D. K. Benjamin, Jr.** 2005. A meta-analysis of medical versus surgical therapy for *Candida* endocarditis. *J. Infect.* **51:** 230–247.
153. **Struijk, D. G., R. T. Krediet, E. W. Boeschoten, P. J. Rietra, and L. Arisz.** 1987. Antifungal treatment of *Candida* peritonitis in CAPD patients. *Lancet* **i:**1375.
154. **Thaler, M., B. Pastakia, T. H. Shawker, T. O'Leary, and P. A. Pizzo.** 1988. Hepatic candidiasis in cancer patients: the evolving picture of the syndrome. *Ann. Intern. Med.* **108:**88–100.
155. **Tortorano, A. M., J. Peman, H. Bernhardt, L. Klingspor, C. C. Kibbler, O. Faure, E. Biraghi, E. Canton, K. Zimmermann, S. Seaton, and R. Grillot.** 2004. Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study. *Eur. J. Clin. Microbiol. Infect. Dis.* **23:**317–322.
156. **Trick, W. E., S. K. Fridkin, J. R. Edwards, R. A. Hajjeh, and R. P. Gaynes.** 2002. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989–1999. *Clin. Infect. Dis.* **35:**627–630.
157. **Venditti, M., F. De Bernardis, A. Micozzi, E. Pontieri, P. Chirletti, A. Cassone, and P. Martino.** 1992. Fluconazole treatment of catheter-related right-sided endocarditis caused by *Candida albicans* and associated with endophthalmitis and folliculitis. *Clin. Infect. Dis.* **14:**422–426.
158. **Viscoli, C., E. Castagnola, M. T. Van Lint, C. Moroni, A. Garaventa, M. R. Rossi, R. Fanci, F. Menichetti, D. Caselli, M. Giacchino, and M. Congiu.** 1996. Fluconazole versus amphotericin B as empirical antifungal therapy of unexplained fever in granulocytopenic cancer patients: a pragmatic, multicentre, prospective and randomised clinical trial. *Eur. J. Cancer* **32A:**814–820.
159. **Viscoli, C., C. Girmenia, A. Marinus, L. Collette, P. Martino, B. Vandercam, C. Doyen, B. Lebeau, D. Spence, V. Krcmery, B. De Pauw, and F. Meunier.** 1999. Candidemia in cancer patients: a prospective, multicenter surveillance study by the Invasive Fungal Infection Group (IFIG) of the European Organization for Research and Treatment of Cancer (EORTC). *Clin. Infect. Dis.* **28:**1071–1079.
160. **Voice, R. A., S. F. Bradley, J. A. Sangeorzan, and C. A. Kauffman.** 1994. Chronic candidal meningitis: an uncommon manifestation of candidiasis. *Clin. Infect. Dis.* **19:**60–66.
161. **Walsh, T. J.** 2002. Echinocandins—an advance in the primary treatment of invasive candidiasis. *N. Engl. J. Med.* **347:**2070–2072.
162. **Walsh, T. J., R. W. Finberg, C. Arndt, J. Hiemenz, C. Schwartz, D. Bodensteiner, P. Pappas, N. Seibel, R. N. Greenberg, S. Dummer, M. Schuster, and J. S. Holcenberg.** 1999. Liposomal amphotericin B for empirical therapy in patients with persistent fever and neutropenia. National Institute of Allergy and Infectious Diseases Mycoses Study Group. *N. Engl. J. Med.* **340:**764–771.
163. **Walsh, T. J., P. Pappas, D. J. Winston, H. M. Lazarus, F. Petersen, J. Raffalli, S. Yanovich, P. Stiff, R. Greenberg, G. Donowitz, M. Schuster, A. Reboli, J. Wingard, C. Arndt, J. Reinhardt, S. Hadley, R. Finberg, M. Laverdiere, J. Perfect, G. Garber, G. Fioritoni, E. Anaissie, and J. Lee.** 2002. Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. *N. Engl. J. Med.* **346:**225–234.
164. **Walsh, T. J., H. Teppler, G. R. Donowitz, J. A. Maertens, L. R. Baden, A. Dmoszynska, O. A. Cornely, M. R. Bourque, R. J. Lupinacci, C. A. Sable, and B. E. dePauw.** 2004. Caspofungin versus liposomal amphotericin B for empirical antifungal therapy in patients with persistent fever and neutropenia. *N. Engl. J. Med.* **351:** 1391–1402.
165. **Walsh, T. J., P. O. Whitcomb, S. G. Revankar, and P. A. Pizzo.** 1995. Successful treatment of hepatosplenic candidiasis through repeated cycles of chemotherapy and neutropenia. *Cancer* **76:**2357–2362.
166. **Warady, B. A., M. Bashir, and L. A. Donaldson.** 2000. Fungal peritonitis in children receiving peritoneal dialysis: a report of the NAPRTCS. *Kidney Int.* **58:**384–389.
167. **Warren, J. W.** 1997. Catheter-associated urinary tract infections. *Infect. Dis. Clin. N. Am.* **11:**609–622.
168. **Weigl, J. A.** 2000. Candida arthritis in a premature infant treated successfully with oral fluconazole for six months. *Ann. Acad. Med. Singapore* **29:**253–255.
169. **Westling, K., A. Thalme, and I. Julander.** 2005. *Candida albicans* tricuspid valve endocarditis in an intravenous drug addict: successful treatment with fluconazole. *Scand. J. Infect. Dis.* **37:**310–311.
170. **Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel.** 1989. Risk factors for hospital-acquired candidemia. A matched case-control study. *Arch. Intern. Med.* **149:**2349–2353.
171. **White, M. H., R. A. Bowden, E. S. Sandler, M. L. Graham, G. A. Noskin, J. R. Wingard, M. Goldman, J. A. van Burik, A. McCabe, J. S. Lin, M. Gurwith, and C. B. Miller.** 1998. Randomized, double-blind clinical trial of amphotericin B colloidal dispersion vs. amphotericin B in the empirical treatment of fever and neutropenia. *Clin. Infect. Dis.* **27:**296–302.
172. **Wiederhold, N. P., and R. E. Lewis.** 2003. The echinocandin antifungals: an overview of the pharmacology, spectrum and clinical efficacy. *Expert Opin. Investig. Drugs* **12:**1313–1333.
173. **Wingard, J. R., M. H. White, E. Anaissie, J. Raffalli, J. Goodman, and A. Arrieta.** 2000. A randomized, double-blind comparative trial evaluating the safety of liposomal amphotericin B versus amphotericin B lipid complex in the empirical treatment of febrile neutropenia. L Amph/ABLC Collaborative Study Group. *Clin. Infect. Dis.* **31:** 1155–1163.
174. **Winston, D. J., J. W. Hathorn, M. G. Schuster, G. J. Schiller, and M. C. Territo.** 2000. A multicenter, randomized trial of fluconazole versus amphotericin B for empiric antifungal therapy of febrile neutropenic patients with cancer. *Am. J. Med.* **108:**282–289.
175. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317.
176. **Wong, P. N., S. K. Mak, K. Y. Lo, G. M. Tong, and A. K. Wong.** 2000. A retrospective study of seven cases of *Candida parapsilosis* peritonitis in CAPD patients: the therapeutic implications. *Perit. Dial. Int.* **20:**76–79.
177. **Zeluff, B. J.** 1990. Fungal pneumonia in transplant recipients. *Semin. Respir. Infect.* **5:**80–89.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch28

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New Developments in Diagnostics and Management of Invasive Candidiasis

SUJATHA KRISHNAN AND LUIS OSTROSKY-ZEICHNER

Until recent years *Candida* spp. were easily disregarded as little more than commensals or contaminants, warranting no treatment or easily controlled by merely removing a Foley catheter or a central line. In the past few decades, there has been a marked increase in the frequency of invasive fungal infections caused by *Candida* spp. (37). The rising incidence of invasive candidiasis is likely related to increasing numbers of immunocompromised patients and lifesaving medical/surgical interventions that prolong life but break down both immunological and physical host defenses.

Candida spp. are the fourth most common cause of all nosocomial bloodstream infections and the third most common cause of nosocomial bloodstream infections in the intensive care unit (ICU) setting in U.S. hospitals (37). Data from a U.S. surveillance study by Wisplinghoff et al. found that *Candida* spp. caused 4.6 bloodstream infections per 10,000 admissions and 9% of all bloodstream infections over a 7-year period (50). Similar trends have been observed in Europe and South America, although estimated incidences vary among different countries (12).

The past two decades have been exciting in the field of medical mycology as new antifungals have been developed. Along with the development of new, effective, and safe therapies, research has shown that early and aggressive therapy is associated with improved survival and better patient outcomes (5, 10).

The development of diagnostics for fungal infections has lagged several decades behind bacteriology and virology (2). Recent research has brought forward interesting markers that appear to be useful not only for diagnosis but also for implementation of novel management strategies and disease monitoring, such as preemptive or empirical therapy. Modern medical mycology goes well beyond traditional treatment of infections, which required a positive culture as a trigger of antifungal therapy. These current strategies (Fig. 1) include prophylaxis and preemptive and empirical therapy (7, 17, 18). These therapeutic strategies can be implemented when a clinical suspicion is supported by diagnostics. The tests can also monitor therapeutic responses. The available tests include serologies focusing on the fungal cell wall components mannan, galactomannan, and (1,3)-β-D-glucan (BG) or antibodies against these antigens, as well as genetic material detection tests. This chapter focuses on these new markers and their use for advanced prevention and treatment strategies.

(1,3)-β-D-GLUCAN

BG is a component of the fungal cell wall of the majority of fungi. Fungi that appear to have lower levels of BG include the zygomycetes and *Cryptococcus* spp. (32). The presence of this compound in patient serum has been used as a routine diagnostic strategy in Asia since the 1980s (29). A chemical cascade related to factor G is used as the basis for its detection. The reaction depends on blood components of horseshoe crabs from the genera *Tachypleus* and *Limulus* (29). A lower cutoff for results (20 versus 60pg/ml for *Limulus*-based testing) has been used for the *Tachypleus*-based kits, as they have an increased affinity to BG (32). The sensitivity and specificity of this test have been reported to be mostly >80% in a variety of patient populations, including immunocompromised hosts and patients on antifungal prophylaxis, with serial and single sample testing (8, 30–32, 34–36, 38, 43). Table 1 summarizes key studies exploring the diagnostic performance of this test. Recently, Japanese investigators have published data on the clinical correlation of BG to postmortem evidence of invasive fungal infections, showing a high degree of correlation and also much higher sensitivity than traditional blood or tissue cultures performed premortem (31). Although limited information exists, it appears that the causative agents of endemic mycosis can also be detected by this test (11). The test has been explored preliminarily with children, and it has been found that baseline levels in neonates are higher than those in adults (46). As such, a different cutoff may be needed in the neonate population. A more recent study done by Zhao et al. (51) showed a sensitivity and a specificity of 81.8 and 82.4%, respectively, in a pediatric population.

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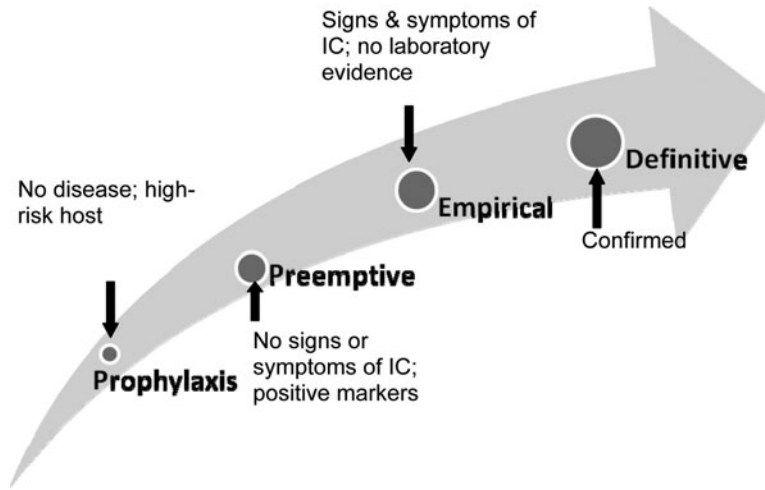

FIGURE 1 Current strategies for the prevention and management of invasive fungal infections. 10.1128/9781555817176.ch28f1

Key studies have shown relatively good sensitivity with limitations in specificity, but remarkably high negative predictive values. Obayashi et al. (29) studied febrile patients, reporting 90% sensitivity and 100% specificity with a cutoff of 20 pg/ml. Ostrosky-Zeichner et al. (34) reported a sensitivity of 69% and specificity of 87% in a multicenter study of single samples from hospitalized patients with proven invasive fungal infections and healthy or hospitalized controls with a cutoff of 60 pg/ml. Pazos et al. (35) reported lower sensitivity but high specificity in ICU patients, most likely in relation to the fungal-infection definitions chosen for this study. This study was also important because it showed that BG levels are not affected by *Candida* colonization. In the recent autopsy series mentioned above, researchers found the sensitivity and specificity of BG to be 95.1 and 85.7%, respectively, with a cutoff of 30 pg/ml; 85.4 and 95.2%, respectively, with a cutoff of 60 pg/ml; and 78.0 and 98.4%, respectively, with a cutoff of 80 pg/ml (30).

When studying BG literature one must be aware of two important issues. (i) As mentioned above, different assays (and therefore studies) have different cutoffs. This is basically related to the affinity of the different assays to BG and does not mean that one assay is more sensitive or potent than the other (32, 34 ,50). Most studies report a proportion of false-positive results, thus affecting the specificities and positive predictive values. Conditions that have been associated with false positives include transfusion of blood products, immunoglobulin infusions, hemodialysis, surgery (specifically the use of surgical gauze), infusion of solutions through filters or membranes, receipt of β-lactam antibiotics, and infection with *Pseudomonas aeruginosa* (13, 14, 19, 22, 27, 33). At least some of the false-positive results may be explained by leaching of BG from membranes or filters (such as the case for hemodialysis) or from materials (as has been postulated for surgical gauze). Along these lines it is also important to mention that BG can detect *Pneumocystis jirovecii* and that researchers believe that at least some of the false-positive results in the studies of invasive fungal infections may be related to undiagnosed *Pneumocystis* infections, as the populations that are at risk of *Pneumocystis* and other invasive fungal infections have significant overlap (1, 20, 26). In the true sense, these should not be actually considered false positives, as the assays are detecting BG from a fungal source, but nevertheless this needs to be considered in the interpretation of test results. Interestingly, and as stated above, colonization with *Candida* spp. does not appear to interfere with the diagnostic performance of the test (35).

Beyond diagnostics, BG has been preliminarily explored for early therapy strategies as part of the continuum of care

TABLE 1 Studies evaluating the diagnostic performance of BG for invasive candidiasis

Author, year (reference)	Sensitivity	Specificity	Population
Mitsutake et al., 1996 (24)	0.84	0.88	Hospitalized patients
Digby et al., 2003 (6)	0.57	0.75	ICU
Odabasi et al., 2004 (32)	0.97	0.93	Hematologic malignancy
Pickering et al., 2005 (38)	0.93	0.77	Hospitalized patients and healthy donors
Ostrosky-Zeichner et al., 2005 (34)	0.78	0.92	Hospitalized patients
Akamatsu et al., 2007 (1)	0.57	0.83	Liver transplant
Persat et al., 2008 (36)	0.85	0.56	ICU and hematologic malignancy
Lunel et al., 2009 (16)	0.64	0.88	Hematologic malignancy
Zhao et al., 2009 (51)	0.82	0.82	Pediatric hematologic malignancy

illustrated in Fig. 1. Takesue et al. (47) retrospectively analyzed the response of fever to fluconazole in patients with a positive BG and multisite colonization by *Candida*, finding that approximately 60% of fevers in patients with a positive BG and colonization in two sites or more responded to the antifungal therapy. Akamatsu et al. (1) also prospectively evaluated 180 liver transplant patients, monitoring BG and starting antifungals for patients with a positive test. They reported a mortality rate of 0.6% for the cohort, which is lower than the mortality rate historically expected for their transplant population. Another interesting use for this agent was reported by Nett et al. (28), who found that BG levels produced during biofilm-forming conditions were 4- to 10-fold higher than those in the planktonic state. Therefore, comparatively high levels of BG in samples drawn through a central venous line as opposed to samples drawn from peripheral sticks may indicate biofilm presence and central line involvement. One can envision a strategy in which BG results drawn both peripherally and through the central venous catheter may be used to make decisions regarding discontinuation of the line, which is a contentious issue in medical mycology.

Studies are beginning to explore if a trend on BG levels correlate with clinical outcomes. In a prospective study done on 52 patients with proven invasive candidiasis per European Organization for Research and Treatment of Cancer/Mycoses Study Group (EORTC/MSG) criteria, serum BG levels were monitored from diagnosis to outcome (45). In this study the BG levels tended to decrease in successfully treated patients and to increase in treatment failures. Further research is required in this regard to establish BG levels as a surrogate for outcomes evaluation in invasive candidiasis, as the effect of sample size and the duration of follow-up is unknown in this study.

Like galactomannan for invasive aspergillosis, BG levels have been included as supportive microbiological evidence of invasive fungal infection in the most recent version of the EORTC/MSG definitions of invasive fungal infections (for research purposes). Japanese guidelines also mention a positive BG and colonization with *Candida* as evidence of possible candidiasis, recommending preemptive antifungal therapy for such patients (30, 48).

MANNAN AND ANTIMANNAN ANTIBODIES

Like BG, mannans are antigens that one could look for in the serum to diagnose invasive candidiasis. Mannan is a large polysaccharide complex with many mannopyranose units linked through α-1,6, α-1,3, α-1,2, or β-1,2 bonds. They are highly immunogenic and are bound noncovalently to the fungal cell wall surface (9). An enzyme immunoassay is commercially available to measure the mannan antigenemia. However, the mannan antigens are rapidly cleared from the bloodstream by host catabolism, and hence the sensitivity of the test is thought to be compromised (39, 41, 42). As antibodies generated against these antigens are highly specific, the use of their levels was incorporated into diagnostic strategies a few years ago. They are marketed as the Platelia *Candida* Ag and Platelia *Candida* Ab. In a study done by Sendid et al., a combination of both the mannan antigen and antibody improved the specificity to 93% and the sensitivity to 80% (41). In another retrospective study done on 96 sera from 26 patients with proven candidiasis, the combination of these two tests had a cumulated specificity of 95%, and positive and negative predicitive values were 79 and 97%, respectively (42). These tests may be limited when the host cannot mount an adequate antibody response to the mannan antigens due to their inherent immunodeficiency, as well as by differences in antibody responses depending on the *Candida* species. These tests are readily available in Europe, and further trials are necessary to assess the value and their pathophysiological relevance.

NEW BIOMARKERS

Aside from BG, which is currently commercially available throughout the world, there is intense research for new biomarkers. This is driven by the fact that current diagnostic methods are less than optimal, by the interest of pharmaceutical companies in finding new surrogate markers for diagnosis and outcomes assessment for drug development trials, and by the recent interest of commercial diagnostic laboratories in these methods. Several molecular techniques focusing on *Candida* DNA detection in blood and tissues have been described. Molecular genetic methods, especially various PCR techniques, are increasingly popular (3, 7, 21, 25). Various modifications of the PCR method are used to detect *Candida* DNA, like real-time PCR (49), quantitative PCR (44), seminested PCR (4), and multiplex-tandem PCR (15). All these tests promise a rapid detection of candidemia and early species identification, which has an impact on antifungal drug selection. However, these are not yet commercially available, nor have they been tested and validated in a multicenter setting. Therefore, more compelling data are warranted prior to routine clinical use.

IMPACT OF BIOMARKERS ON IC MANAGEMENT

Beyond conventional therapy, which involves a positive culture and "reactive" antifungal therapy, new preventive and early therapy approaches have surfaced (Fig. 1). Antifungal prophylaxis is an attractive strategy, as the best way to deal with a fungal infection is to prevent it from ever occurring. For prophylaxis to be cost-effective and risk balanced, one must select a population at high risk for the disease, as this strategy involves the administration of prophylactic antifungals to a potentially large population. While diagnostics do not play a role in selecting the population for this intervention, they may be useful in excluding underlying active disease at baseline (which would naturally switch the focus from prophylaxis to preemptive therapy) and to detect failures or "breakthroughs."

Preemptive antifungal therapy is the approach that is most dependent on biomarkers. Preemptive therapy assumes that periodic surveillance with an early diagnostic method is being conducted at regular intervals in a high-risk patient. When disease is detected through a positive biomarker, antifungal therapy is triggered. This therapy is then continued for the typical duration of treatment for the disease, or until the marker is cleared.

Empirical therapy again includes a high-risk patient, who in this case is displaying signs and symptoms of infection in a setting where other causes of these have been excluded, and traditional fungal cultures are pending or negative. Aware of the poor performance of current diagnostic methods and the reduced mortality rate associated with early therapy, clinicians typically start empirical antifungal therapy. Although unproven, this is a widespread practice. There is a single report of a randomized, placebo-controlled trial of empirical therapy in which no significant differences were noted for fluconazole and placebo arms in a composite

endpoint (resolution of fever; absence of invasive fungal infection; no discontinuation because of toxicity; and no need for a nonstudy, systemic antifungal medication) (40). As mentioned in this study, future trials should focus on systematically enrolling higher-risk hosts and considering different endpoints, such as mortality or length of stay. The use of new diagnostics within empirical therapy could be focused on confirming or excluding the infection, thus being a primary driver for the continuation or discontinuation of antifungal therapy.

CONCLUSIONS

Invasive candidiasis is an infection of increasing prevalence which is associated with high morbidity and mortality. Traditional diagnostics have less-than-optimal sensitivity, and recent developments in the field have prompted the development of new diagnostics and markers. BG is a biomarker with reasonable sensitivity and specificity for the diagnosis of invasive fungal infections. Although diagnostic performance varies from study to study, results are generally consistent and the test appears to have high sensitivity and a high negative predictive value, making it a valuable test in excluding the presence of invasive fungal infection. Other markers such as *Candida* mannan antigen and antibody are accumulating data and seem to be very effective. The ultimate frontier is nucleic acid detection, which is not standardized or commercially available at this point.

Having reliable diagnostics is of great significance to medical mycology, as they will not only allow for faster and more reliable diagnosis of invasive candidiasis but also provide access to advanced management strategies, such as preemptive and empirical therapy. Another important use of these markers will be therapeutic monitoring and outcome evaluation (23).

REFERENCES

1. **Akamatsu, N., Y. Sugawara, J. Kaneko, S. Tamura, and M. Makuuchi.** 2007. Preemptive treatment of fungal infection based on plasma (1 → 3)beta-D-glucan levels after liver transplantation. *Infection* **35:**346–351.
2. **Alexander, B. D.** 2002. Diagnosis of fungal infection: new technologies for the mycology laboratory. *Transpl. Infect. Dis.* **4**(Suppl. 3)**:**32–37.
3. **Arishima, T., and J. Takezawa.** Use of PCR based diagnosis for common invasive fungal infections in the intensive care unit. *Nippon Ishinkin Gakkai Zasshi* **47:**283–288. (In Japanese.)
4. **Cerikcioglu, N., B. Aksu, T. D. Dal, U. Deniz, H. S. Bilgen, E. Ozek, and G. Söyletir.** 2010. Seminested PCR for detection and identification of *Candida* species directly from blood culture bottles. *New Microbiol.* **33:**57–62.
5. **Chamilos, G., R. E. Lewis, and D. P. Kontoyiannis.** 2008. Delaying amphotericin B-based frontline therapy significantly increases mortality among patients with hematologic malignancy who have zygomycosis. *Clin. Infect. Dis.* **47:** 503–509.
6. **Digby, J., J. Kalbfleisch, A. Glenn, A. Larsen, W. Browder, and D. Williams.** 2003. Serum glucan levels are not specific for presence of fungal infections in intensive care unit patients. *Clin. Diagn. Lab. Immunol.* **10:**882–885.
7. **Einsele, H., and J. Loeffler.** 2008. Contribution of new diagnostic approaches to antifungal treatment plans in high-risk haematology patients. *Clin. Microbiol. Infect.* **14**(Suppl. 4)**:**37–45.
8. **Ellis, M., B. Al-Ramadi, M. Finkelman, U. Hedstrom, J. Kristensen, H. Ali-Zadeh, and U. Hedstrom.** 2008. Assessment of the clinical utility of serial beta-D-glucan concentrations in patients with persistent neutropenic fever. *J. Med. Microbiol.* **57**(Pt. 3)**:**287–295.
9. **Fukazawa, Y.** 1989. Antigenic structure of *Candida albicans*. Immunochemical basis of the serologic specificity of the mannans in yeasts. *Immunol. Ser.* **47:**37–62.
10. **Garey, K. W., M. Rege, M. P. Pai, D. E. Mingo, K. J. Suda, R. S. Turpin, and D. T. Bearden.** 2006. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. *Clin. Infect. Dis.* **43:**25–31.
11. **Girouard, G., C. Lachance, and R. Pelletier.** 2007. Observations on (1-3)-beta-D-glucan detection as a diagnostic tool in endemic mycosis caused by *Histoplasma* or *Blastomyces*. *J. Med. Microbiol.* **56**(Pt. 7)**:**1001–1002.
12. **Guery, B. P., M. C. Arendrup, G. Auzinger, E. Azoulay, M. Borges Sa, E. M. Johnson, E. Müller, C. Putensen, C. Rotstein, G. Sganga, M. Venditti, R. Zaragoza Crespo, and B. J. Kullberg.** 2009. Management of invasive candidiasis and candidemia in adult non-neutropenic intensive care unit patients. Part I. Epidemiology and diagnosis. *Intensive Care Med.* **35:**55–62.
13. **Kanamori, H., K. Kanemitsu, T. Miyasaka, K. Ameku, S. Endo, T. Aoyagi, K. Inden, M. Hatta, N. Yamamoto, H. Kunishima, H. Yano, K. Kaku, Y. Hirakata, and M. Kaku.** 2009. Measurement of (1-3)-beta-D-glucan derived from different gauze types. *Tohoku J. Exp. Med.* **217:** 117–121.
14. **Kedzierska, A.** 2007. (1→3)-Beta-D-glucan—a new marker for the early serodiagnosis of deep-seated fungal infections in humans. *Pol. J. Microbiol.* **56:**3–9.
15. **Lau, A., C. Halliday, S. C. Chen, E. G. Playford, K. Stanley, and T. C. Sorrell.** 2010. Comparison of whole blood, serum, and plasma for early detection of candidemia by multiplex-tandem PCR. *J. Clin. Microbiol.* **48:** 811–816.
16. **Lunel, F. M., M. A. Mennink-Kersten, D. Ruegebrink, H. A. van der Lee, J. P. Donnelly, N. M. Blijlevens, and P. E. Verweij.** 2009. Value of *Candida* serum markers in patients with invasive candidiasis after myeloablative chemotherapy. *Diagn. Microbiol. Infect. Dis.* **64:**408–415.
17. **Maertens, J., D. Deeren, D. Dierickx, and K. Theunissen.** 2006. Preemptive antifungal therapy: still a way to go. *Curr. Opin. Infect. Dis.* **19:**551–556.
18. **Maertens, J., K. Buve, K. Theunissen, W. Meersseman, E. Verbeken, G. Verhoef, J. Van Eldere, and K. Lagrou.** 2009. Galactomannan serves as a surrogate endpoint for outcome of pulmonary invasive aspergillosis in neutropenic hematology patients. *Cancer* **115:**355–362.
19. **Marty, F. M., C. M. Lowry, S. J. Lempitski, D. W. Kubiak, M. A. Finkelman, and L. R. Baden.** 2006. Reactivity of (1→3)-beta-D-glucan assay with commonly used intravenous antimicrobials. *Antimicrob. Agents Chemother.* **50:**3450–3453.
20. **Marty, F. M., S. Koo, J. Bryar, and L. R. Baden.** (1→3)beta-D-glucan assay positivity in patients with *Pneumocystis* (*carinii*) *jiroveci* pneumonia. *Ann. Intern. Med.* **147:**70–72.
21. **McMullan, R., L. Metwally, P. V. Coyle, S. Hedderwick, B. McCloskey, H. J. O'Neill, C. C. Patterson, G. Thompson, C. H. Webb, and R. J. Hay.** 2008. A prospective clinical trial of a real-time polymerase chain reaction assay for the diagnosis of candidemia in nonneutropenic, critically ill adults. *Clin. Infect. Dis.* **46:**890–896.
22. **Mennink-Kersten, M. A., D. Ruegebrink, and P. E. Verweij.** 2008. *Pseudomonas aeruginosa* as a cause of 1,3-beta-D-glucan assay reactivity. *Clin. Infect. Dis.* **46:**1930–1931.

23. **Miceli, M. H., M. L. Grazziutti, G. Woods, W. Zhao, M. H. Kocoglu, B. Barlogie, and E. Anaissie.** 2008. Strong correlation between serum *Aspergillus* galactomannan index and outcome of aspergillosis in patients with hematological cancer: clinical and research implications. *Clin. Infect. Dis.* **46:**1412–1422.
24. **Mitsutake, K., T. Miyazaki, T. Tashiro, Y. Yamamoto, H. Kakeya, T. Otsubo, S. Kawamura, M. A. Hossain, T. Noda, Y. Hirakata, and S. Kohno.** 1996. Enolase antigen, mannan antigen, Cand-Tec antigen, and beta-glucan in patients with candidemia. *J. Clin. Microbiol.* **34:**1918–1921.
25. **Morace, G., L. Pagano, M. Sanguinetti, B. Posteraro, L. Mele, F. Equitani, G. D'Amore, G. Leone, and G. Fadda.** 1999. PCR-restriction enzyme analysis for detection of *Candida* DNA in blood from febrile patients with hematological malignancies. *J. Clin. Microbiol.* **37:** 1871–1875.
26. **Nakamura, H., M. Tateyama, D. Tasato, S. Haranaga, S. Yara, F. Higa, Y. Ohtsuki, and J. Fujita.** 2009. Clinical utility of serum beta-D-glucan and KL-6 levels in *Pneumocystis jirovecii* pneumonia. *Intern. Med.* **48:**195–202.
27. **Nakao, A., M. Yasui, T. Kawagoe, H. Tamura, S. Tanaka, and H. Takagi.** 1997. False-positive endotoxemia derives from gauze glucan after hepatectomy for hepatocellular carcinoma with cirrhosis. *Hepatogastroenterology* **44:** 1413–1418.
28. **Nett, J., L. Lincoln, K. Marchillo, and D. Andes.** 2007. Beta-1,3 glucan as a test for central venous catheter biofilm infection. *J. Infect. Dis.* **195:**1705–1712.
29. **Obayashi, T., H. Tamura, S. Tanaka, M. Ohki, S. Takahashi, M. Arai, M. Masuda, and T. Kawai.** 1985. A new chromogenic endotoxin-specific assay using recombined *Limulus* coagulation enzymes and its clinical applications. *Clin. Chim. Acta* **149:**55–65.
30. **Obayashi, T., K. Negishi, T. Suzuki, and N. Funata.** 2008. Reappraisal of the serum (1→3)-beta-D-glucan assay for the diagnosis of invasive fungal infections—a study based on autopsy cases from 6 years. *Clin. Infect. Dis.* **46:** 1864–1870.
31. **Obayashi, T., M. Yoshida, T. Mori, H. Goto, A. Yasuoka, H. Iwasaki, and A. Ito.** 1995. Plasma (1→3)-beta-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. *Lancet* **345:**17–20.
32. **Odabasi, Z., G. Mattiuzzi, E. Estey, H. Kantarjian, F. Saeki, R. J. Ridge, P. A. Ketchum, M. A. Finkelman, J. H. Rex, and L. Ostrosky-Zeichner.** 2004. Beta-D-glucan as a diagnostic adjunct for invasive fungal infections: validation, cutoff development, and performance in patients with acute myelogenous leukemia and myelodysplastic syndrome. *Clin. Infect. Dis.* **39:**199–205.
33. **Ohata, A., M. Usami, T. Horiuchi, K. Nagasawa, and K. Kinoshita.** 2003. Release of (1→3)-beta-D-glucan from depth-type membrane filters and their in vitro effects on proinflammatory cytokine production. *Artif. Organs* **27:** 728–735.
34. **Ostrosky-Zeichner, L., B. D. Alexander, D. H. Kett, J. Vazquez, P. G. Pappas, F. Saeki, P. A. Ketchum, J. Wingard, R. Schiff, H. Tamura, M. A. Finkelman, and J. H. Rex.** 2005. Multicenter clinical evaluation of the (1→3) beta-D-glucan assay as an aid to diagnosis of fungal infections in humans. *Clin. Infect. Dis.* **41:**654–659.
35. **Pazos, C., J. Ponton, and A. Del Palacio.** 2005. Contribution of (1→3)-beta-D-glucan chromogenic assay to diagnosis and therapeutic monitoring of invasive aspergillosis in neutropenic adult patients: a comparison with serial screening for circulating galactomannan. *J. Clin. Microbiol.* **43:** 299–305.
36. **Persat, F., S. Ranque, F. Derouin, A. Michel-Nguyen, S. Picot, and A. Sulahian.** 2008. Contribution of the (1→3)-beta-D-glucan assay for diagnosis of invasive fungal infections. *J. Clin. Microbiol.* **46:**1009–1013.
37. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
38. **Pickering, J. W., H. W. Sant, C. A. Bowles, W. L. Roberts, and G. L. Woods.** 2005. Evaluation of a (1→3)-beta-D-glucan assay for diagnosis of invasive fungal infections. *J. Clin. Microbiol.* **43:**5957–5962.
39. **Poulain, D., R. Robert, F. Mesnard, B. Sendid, G. Lepage, and D. Camus.** 1997. Clearances of *Candida albicans*-derived alpha- and beta-linked mannose residues in sera from patients with candidiasis. *Eur. J. Clin. Microbiol. Infect. Dis.* **16:**16–20.
40. **Schuster, M. G., J. E. Edwards, Jr., J. D. Sobel, R. O. Darouiche, A. W. Karchmer, S. Hadley, G. Slotman, H. Panzer, P. Biswas, and J. H. Rex.** 2008. Empirical fluconazole versus placebo for intensive care unit patients: a randomized trial. *Ann. Intern. Med.* **149:**83–90.
41. **Sendid, B., M. Tabouret, J. L. Poirot, D. Mathieu, J. Fruit, and D. Poulain.** 1999. New enzyme immunoassays for sensitive detection of circulating *Candida albicans* mannan and antimannan antibodies: useful combined test for diagnosis of systemic candidiasis. *J. Clin. Microbiol.* **37:** 1510–1517.
42. **Sendid, B., J. L. Poirot, M. Tabouret, A. Bonnin, D. Caillot, D. Camus, and D. Poulain.** 2002. Combined detection of mannanaemia and antimannan antibodies as a strategy for the diagnosis of systemic infection caused by pathogenic *Candida* species. *J. Med. Microbiol.* **5:** 433–442.
43. **Senn, L., J. O. Robinson, S. Schmidt, M. Knaup, N. Asahi, S. Satomura, S. Matsuura, B. Duvoisin, J. Bille, T. Calandra, and O. Marchetti.** 2008. 1,3-Beta-D-glucan antigenemia for early diagnosis of invasive fungal infections in neutropenic patients with acute leukemia. *Clin. Infect. Dis.* **46:**878–885.
44. **Shao, Q., L. Gao, L. L. Wang, Y. Ding, H. Yang, J. J. Suo, D. X. Shen, and L. Yu.** 2010. Quantitative PCR for diagnosis of invasive fungal infections in patients with hematologic malignancies. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* **18:**499–504. (In Chinese.)
45. **Sims, C. R., J. Mohr, V. Paetznick, J. Rodriguez, M. Finkelman, and L. Ostrosky- Zeichner.** 2006. Correlation of clinical outcomes with BG levels in patients with invasive candidiasis (IC), abstr. M-1599. *46th Intersci. Conf. Antimicrob. Agents Chemother.* American Society for Microbiology, Washington, DC.
46. **Smith, P. B., D. K. Benjamin, Jr., B. D. Alexander, M. D. Johnson, M. A. Finkelman, and W. J. Steinbach.** 2007. Quantification of 1,3-beta-D-glucan levels in children: preliminary data for diagnostic use of the beta-glucan assay in a pediatric setting. *Clin. Vaccine Immunol.* **14:** 924–925.
47. **Takesue, Y., M. Kakehashi, H. Ohge, Y. Imamura, Y. Murakami, M. Sasaki, M. Morifuji, Y. Yokoyama, M. Kouyama, T. Yokoyama, and T. Sueda.** 2004. Combined assessment of beta-D-glucan and degree of *Candida* colonization before starting empiric therapy for candidiasis in surgical patients. *World J. Surg.* **28:**625–630.
48. **Tsuruta, R., H. Mizuno, T. Kaneko, Y. Oda, K. Kaneda, M. Fujita, T. Inoue, S. Kasaoka, and T. Maekawa.** 2007. Preemptive therapy in nonneutropenic patients with *Candida* infection using the Japanese guidelines. *Ann. Pharmacother.* **41:**1137–1143.
49. **Wellinghausen, N., D. Siegel, J. Winter, and S. Gebert.** 2009. Rapid diagnosis of candidaemia by real-time PCR detection of *Candida* DNA in blood samples. *J. Med. Microbiol.* **58**(Pt. 8)**:**1106–1111.

50. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317.

51. **Zhao, L., J. Y. Tang, Y. Wang, Y. F. Zhou, J. Chen, B. R. Li, and H. L. Xue.** 2009. Value of plasma beta-glucan in early diagnosis of invasive fungal infection in children. *Zhongguo Dang Dai Er Ke Za Zhi* **11:**905–908. (In Chinese.)

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch29

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The Epidemiology of Invasive Candidiasis

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Candida species are the most common cause of invasive fungal infections (IFI; those of blood, normally sterile body fluids, deep tissue, and organs). Invasive *Candida* infections are almost always health care-associated infections (HAI) and may be either nosocomial (onset after 48 to 72 h of hospitalization) or community onset (CO) (occurring ≤2 days of admission) (110, 199). Contributing factors include exposure to broad-spectrum antibacterial agents, corticosteroids, cytotoxic chemotherapy, and prolonged use of intravascular catheters (Table 1).

Candida spp., led by *Candida albicans*, accounted for 88% of all nosocomial fungal infections in the United States between 1980 and 1990 (101, 102). More recently, a multicenter survey conducted in the United States between 2004 and 2008 found that *Candida* spp. accounted for 75% of IFI in hospitalized patients, although the proportion of IFI due to *Candida* varied from 31.6% (hematologic stem cell transplantation [HSCT]) to 96.3% (neonatal intensive care unit [NICU]) according to the clinical service and underlying condition of the patient (93, 94, 166, 167). Between 1995 and 2002, the frequency of nosocomial candidemia in U.S. hospitals rose significantly, from 8 to 12% of all reported bloodstream infections (BSI) (300). Clearly, HAI due to *Candida* spp. constitute a major public health problem (218).

This chapter discusses the epidemiological profile of candidemia and invasive candidiasis (IC) worldwide as well as risk factors for infection with selected species. The susceptibility of the various species of *Candida* to antifungal agents is also discussed.

INCIDENCE OF CANDIDEMIA AND IC

Candida species are the fourth leading cause of nosocomial BSI in the United States, accounting for 8 to 12% of all BSI acquired in the hospital (300). Estimating the true incidence of either candidemia or IC (involving not only BSI but also other deep infections not necessarily associated with candidemia) has been difficult due to the fact that these infections are not reportable in most regions and/or countries of the world. Consequently, the best estimates of incidence have been derived from billing codes and hospital discharge surveys (218, 297, 309) and from population-based surveillance (3, 83, 105).

Zilberberg et al. (310) used billing codes to study the secular trends in candidemia-related hospitalization among adults in the United States and found that the incidence of candidemia rose by 52% between 2000 and 2005 (Table 2). Similar increases in incidence were seen among all age groups; however, there was approximately a 10-fold difference in the candidemia-related hospitalization incidence between the youngest group (1.5 to 2.3 cases per 100,000 population among those aged 18 to 44 years) and the oldest group (17.3 to 25.0 cases per 100,000 population among those aged at least 85 years) throughout the study period (Table 2). In an analysis of regional patterns of antimicrobial-resistant infections, Zilberberg et al. (311) reported an increasing incidence of IC (candidemia and other deep-seated infections, including candidiasis of the lung, disseminated candidiasis, candidal endocarditis, meningitis, esophagitis, and enteritis) in all four geographic regions (Northeast, Midwest, South, and West) of the United States, with the highest incidence of *Candida* hospitalizations (19 to 26 infections per 100,000 population) observed in the South (Table 3).

These data lend support to the earlier findings of Wilson et al. (297) and Pfaller and Diekema (218), who used National Hospital Discharge Survey data to show that estimates of IC incidence were steady or increased between 1996 and 2003 at 22 to 29 infections per 100,000 population. Given the fact that these surveys include not only candidemia but also other forms of IC that may not be associated with positive blood cultures, the estimates may be higher than other reports of candidemia incidence (199).

The data in Tables 2 and 3 compare favorably with the findings of U.S. population-based studies conducted by the Centers for Disease Control and Prevention (CDC) in 1998 to 2000 (24 infections/100,000/year) and 2008 to 2009 (25 infections/100,000/year) in the Baltimore, MD, metropolitan area (Table 4) (3, 83). By comparison, population-based studies in the San Francisco, CA, and Atlanta, GA, metro-

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TABLE 1 Candidemia risk factors for hospitalized patients[a]

Risk factor	Possible role in infection
Antimicrobial agents (number and duration)[b]	Provide vascular access and promote fungal colonization
Adrenal corticosteroid	Immunosuppression
Age (<1 yr, >70 yr)	Immunosuppression
Chemotherapy[b]	Immunosuppression Mucosal disruption
Malignancy	Immunosuppression
Previous colonization[b]	Translocation across mucosa
Gastric acid suppression	Colonization and translocation
Indwelling catheter[b] Central venous catheter Pressure transducer	Direct vascular access Contaminated product
Total parenteral nutrition	Direct vascular access Hyperglycemia Contamination of infusate
Neutropenia (<500/mm^3)[b]	Immunosuppression
Surgery (GI)	Route of infection Mucosal disruption Direct vascular access
Mechanical ventilation	Route of infection
Renal failure/hemodialysis[b]	Route of infection Immunosuppression
Malnutrition	Immunosuppression
Hospital or ICU stay	Exposure to pathogens Exposure to additional risk factors
Severity of underlying illness	Immunosuppression Invasive procedures

[a]Data compiled from references 26, 59, 165, 176, 178, 179, 216, 233, 235, and 295.

[b]Independent risk factor.

politan areas and in the states of Iowa and Connecticut report lower annual incidence rates, 6 to 14 per 100,000 (Table 4) (3, 58, 83, 105). Notably, the incidence of IC in Atlanta, GA, increased from 8.7/100,000 in 1992 to 1993 (83) to 14/100,000 in 2008 to 2009 (3). Given these data, it is clear that the overall burden of disease due to IC in the United States is substantial and has not decreased over the past two decades.

In addition to population-based surveillance of candidemia and IC in the United States, several similarly designed studies have been conducted in Australia (27), northern Europe (9, 10, 12, 232, 256), Scotland (174), Spain (7, 196), and Canada (118) (Table 4). With the exception of Denmark (9, 10), the incidence in Australia, Europe, and Canada was considerably lower than that reported from the United States, although in most of these studies an increase in incidence was documented over the course of the study. Although variation in study design may account for some differences, numerous factors may also contribute to the variation in incidence throughout the world, including differences in patient demographics and comorbidities, as well as differences in medical practice, especially the use of long-term intravascular catheters, and antibacterial and antifungal usage practices (7, 127, 256). Likewise, the frequency of diagnostic test ordering, especially of blood cultures, and the type of blood culture systems employed may affect the incidence rates in laboratory-based surveillance (10, 95, 256). Despite the variation in incidence found among the different population-based studies, the rate of IC is far higher than that of any other invasive mycosis and exceeds that of many invasive bacterial diseases (55, 66, 67, 118, 192, 218, 309).

Population-based surveillance also provides the opportunity to define the incidence of IC in specific risk groups. In every one of the population-based studies of candidemia that have examined incidence in different age groups, the highest incidence of infection occurred at the extremes of the age spectrum (Table 5). The highest rates of candidemia in infants less than 1 year of age and in adults over the age of 65 have been reported from two U.S. studies conducted by the CDC (83, 105) (Table 5). Whereas a study from the United Kingdom found higher rates of candidemia in males <1 year and ≥65 years than in females (96), the U.S. studies examined the influence of race on the incidence of candidemia and found that the incidence was at least fourfold higher among blacks versus whites at every age group (Table 5). Kao et al. (105) found a remarkably high incidence, 466 infections per 100,000 neonates (<30 days of age), which was much higher among black neonates (960 per 100,000) than white neonates (238 per 100,000). The discrepancy between rates of infection in black and white infants may be due in part to the elevated incidence of prematurity and low birth weight among black infants (83, 105). Interestingly, a recent CDC study of candidemia in U.S. NICUs found that while there was a decrease in the overall incidence of candidemia among neonates weighing less than 1,000 g in the United States between 1995 and 2004, there was a great variation in the rates of candidemia across NICUs, suggesting that significant differences exist among neonates cared for in these various NICUs that are not adjusted for when evaluating incidence based on birth weight category (70). Additional study of institutional differences between low- and high-incidence NICUs may be helpful in identifying risk factors, infection control measures, or prophylaxis practices in very-low-birth-weight infants (70).

TABLE 2 Population incidence of candidemia in the United States by age group, 2001 to 2005[a]

	No. of cases/100,000 population/year				
Age group (yr)	2001	2002	2003	2004	2005
All ages	3.7				5.6
18–44	1.5	1.8	2.2	2.3	2.0
45–64	5.1	6.0	6.2	6.7	6.8
65–84	14.2	15.5	16.8	16.5	18.6
≥85	17.3	19.5	21.5	21.9	25.0

[a]Data compiled from references 227 and 310.

TABLE 3 Population incidence of IC in the United States by region, 2000 to 2005[a]

Year	No. of infections/100,000 population by region[b]			
	NE	MW	South	West
2000	17	15	20	11
2001	20	15	19	12
2002	16	15	21	13
2003	18	17	22	12
2004	18	16	23	12
2005	19	18	26	14

[a]Data compiled from reference 311.
[b]NE, Northeast; MW, Midwest.

Population-based surveillance studies have also documented a high incidence of candidemia among cancer patients (71 per 100,000) and adults with diabetes (28 per 100,000), as well as the near universality of central venous catheters among patients diagnosed with candidemia (3, 7, 10, 83, 105). Regarding the latter observation, it is noteworthy that the formation of biofilms on central venous catheters by *Candida* spp. is associated with nosocomial infections (237). These studies also demonstrate that candidemia is no longer found primarily within the intensive care unit (ICU), as fewer than 40% of patients in most studies were in an ICU at the time of diagnosis (Table 6) (7, 83, 105, 271).

SHIFT IN CANDIDEMIA OCCURRENCE AND THE EMERGENCE OF CO INFECTION

An increasing incidence of IC overall, combined with data from the National Nosocomial Infection System survey, which found a decline in the frequency of candidemia among ICU patients in the United States (283), suggests that the burden of IC is shifting from the ICU to the general hospital and even outpatient settings (83, 110, 271). Indeed, Hajjeh et al. (83) have shown that in 1998 through 2000, only 36% of *Candida* BSIs occurred in the ICU, whereas 28% were CO in nature, an increase of almost 10% over that reported in 1992 and 1993 (20% CO) (Table 6). Subsequently, Sofair et al. (271) determined that 31% of 1,143 cases of candidemia were CO infections (occurring ≤2 days after hospital admission), leading participants in a recent HAI summit to conclude that clinicians should be aware of the potential for *Candida* to be a cause of BSI in patients presenting to the emergency department (110). A majority of the HAI summit panelists and Infectious Diseases Society of America members responding to a web-based survey supported the concept that "patients with serious HAIs who have risk factors for fungal infections require early empiric antifungal therapy to reduce mortality," further underscoring the recognized importance of IC occurring both inside and outside of hospital settings (110).

Recently, Shorr et al. (266) hypothesized that early-onset (CO) candidemia, defined as a positive blood culture taken at or within 2 days of hospital admission, might represent a distinct clinical entity associated with substantial morbidity and mortality. To examine this hypothesis and to assess the epidemiology of CO candidemia, they retrospectively reviewed the Cardinal Health Outcomes Research Database, comprising all acute-care admission to 176 participating U.S. hospitals between 2000 and 2005 (266). They found that compared with patients with other types of CO BSI, patients with candidemia were younger (mean age, 68 years versus 72 and 75 years for patients with gram-positive and gram-negative bacteremia, respectively), yet were more severely ill at presentation and were more likely to have been

TABLE 4 Estimated incidence of candidemia: population-based studies in Europe, Australia, Canada, and the United States

Region	Years	Location	No. of cases/ 100,000/yr	Reference
Europe	1995–1999	Finland	1.9	232
	1991–1994	Norway	2.0	256
	1995–1999	Norway	3.0	256
	2005–2008	United Kingdom	3.3	96
	1997–1999	Spain	3.5	196
	2002–2003	Spain	4.9	7
	2005–2006	Scotland	4.8	174
	1995–1999	Iceland	4.9	12
	2003–2004	Denmark	11.0	9
	2004–2006	Denmark	11.0	10
Asia-Pacific	2001–2004	Australia	1.8	27
North America	1999–2004	Canada	2.8	118
	1998–2001	Iowa	6.0	58
	1992–1993	California	7.1	105
	1992–1993	Georgia	8.7	105
	2008–2009	Georgia	14.0	3
	1998–2000	Connecticut	7.1	83
	1998–2000	Maryland	24.0	83
	2008–2009	Maryland	25.0	3

TABLE 5 Highest incidences of candidemia in the youngest and oldest patients groups: population based surveillance

Study location	Year(s)	No. of cases/100,000/year[a]			Reference
		Total	Patients <1 yr old	Patients ≥65 yr old	
Finland	1995–1999	1.9	9.4	5.2	232
Norway	1991–2003	2.4	10.3	7.0	256
Iceland	1995–1999	4.9	11.3	19.0	12
Spain	2002–2003	4.9	38.8	12.0	7
United Kingdom	2008	3.2 (all)	16.1 (M)	13.0 (M)	96
			11.0 (F)	7.0 (F)	96
Canada	1999–2004	2.8	19.0	15.0	118
United States	1992–1993	8.0 (all)	75.0 (all)	26.0 (all)	105
		6.0 (W)	41.0 (W)	20.0 (W)	105
		12.0 (B)	165.0 (B)	40.0 (B)	105
United States	1998–2000	10.0 (all)	37.0 (W)	37.0 (W)	83
			160.0 (B)	100.0 (B)	83

[a]M, male; F, female; W, white patients only; B, black patients only.

recently hospitalized. Recent discharge from an acute-care hospital was reported for 37.4% and admission from another health care facility or nursing home for 38.1%. The risk adjusted mortality rate, length of stay (LOS), and affordable cost were greater for CO candidemia patients than for patients with CO bacteremia (Table 7). Furthermore, the rate of CO *Candida* infections appeared to increase over the period of study. Thus, candidemia has spread beyond the confines of acute-care hospitals and early-onset/CO candidemia should be considered a distinct clinical entity with unique implications for both infectious morbidity and mortality (266). Clinicians are urged to be vigilant and to consider candidemia in patients with a likely BSI and multiple risk factors for an HAI (110, 266). Failure to consider this infection in at-risk subjects may have adverse consequences.

It is clear that as cost pressures continue to mount and hospital discharges to secondary care facilities (including home care) are expedited, the boundaries between nosocomial and CO infection may no longer be valid. In the United States, ever-increasing numbers of patients are managed in the community with risk-increasing procedures such as chemotherapy, dialysis, parenteral nutrition, parenteral antibiotics, and indwelling catheters (50, 110, 271). Given these considerations, it is interesting that the proportion of CO candidemia in Spain (11%) was lower than in the United States (28%) (Table 6), possibly due to a difference in outpatient central venous catheter use and the increasing practice in the United States of the management of various chronic diseases at home rather than in the hospital (7, 83).

The recognition of CO candidemia raises the issue of whether an antifungal therapy should be added to the first-line empirical antimicrobial regimen in patients presenting with sepsis (50, 110). It is now apparent that a delay in initiating antifungal therapy in the critically ill patient is associated with worse outcomes (an increase in the mortality rate of 8% for each hour in which appropriate therapy is delayed) and potentially devastating complications such as endophthalmitis, endocarditis, osteomyelitis, and disseminated candidiasis (74, 115, 164, 189). Ideally, such therapeutic decisions should be guided by rapid diagnostic markers and/or risk stratification schema in order to maximize benefits and decrease the risk of drug toxicity and resistance development. In the absence of such aids, it seems reasonable to consider treating high-risk patients hospitalized with clinical evidence of sepsis with an antifungal agent with systemic activity against *Candida* spp., especially if they are known to be colonized with a yeast (50, 110, 266). Presently, it is notable that the median times to the institution of effective antimicrobial therapy in septic shock are 5.5 h for patients with bacterial infections and 31.5 h for those with infections due to *Candida* (74, 115, 164). These differences highlight the hesitancy of clinicians to institute early antifungal therapy.

TABLE 6 Changes in the distribution of *Candida* BSI: emergence of CO candidemia

Study location (reference)	No. of BSI	% of *Candida* BSI by hospital location		
		CO	ICU	Inpatient
Spain (7)	345	11	33	56
United States (105)	772	20	NA[a]	80
United States (83)	1,143	28	36	36
Australia (27)	1,005	19	38	44

[a]NA, not available.

TABLE 7 Burden of early-onset candidemia in the United States[a]

Parameter	Type of BSI[b]		
	Candida	GM-pos	GM-neg
Mortality (%)	28.3	20.0	10.0
LOS (days)	13.7	10.6	8.0
Cost ($)	30,219	18,526	12,305

[a]Data compiled from reference 266.
[b]Abbreviations: GM-pos, gram positive; GM-neg, gram negative.

RESERVOIRS FOR CANDIDEMIA AND IC

The predominant source of infection due to *Candida* spp., from superficial mucosal and cutaneous disease to hematogenous dissemination, is a patient's own flora. That is, most types of candidiasis represent *endogenous* infection in which the normally commensal host flora takes advantage of the "opportunity" to cause infection (200). In order to do so, there must be a lowering of the host's anti-*Candida* barrier (Table 1). In cases of *Candida* BSI, transfer of the organism from the gastrointestinal (GI) mucosa to the bloodstream requires prior overgrowth of the numbers of yeasts in their commensal habitat coupled with a breach in the integrity of the GI mucosa (2, 63, 145, 184).

Exogenous acquisition, or transmission, of *Candida* may also account for a proportion of certain types of candidiasis (13). Examples of vehicles which may introduce *Candida* into the human host include the use of contaminated irrigation solutions, parenteral nutrition fluids, vascular pressure transducers, cardiac valves, and corneas (184). Transmission of *Candida* spp. from health care workers to patients and from patient to patient has been well documented, especially for the ICU environment (13, 24, 151). The hands of health care workers serve as potential reservoirs of nosocomial transmission of *Candida* spp. (59, 123, 204, 273, 287).

GLOBAL TRENDS IN SPECIES DISTRIBUTION AMONG CANDIDA ISOLATES FROM CANDIDEMIA AND IC

Among the more than 100 species of *Candida* that have been described, 20 to 30 have been implicated in clinical infections (Table 8). *C. albicans* is the species most commonly

TABLE 8 Species distribution of *Candida* isolates from patients with IC: ARTEMIS Global Surveillance Program, 2001 to 2007[a,b]

Species	% of total by year (no. tested)						
	2001 (21,804)	2002 (24,680)	2003 (33,106)	2004 (33,406)	2005 (28,412)	2006 (29,167)	2007 (31,078)
C. albicans	65.4	61.4	62.3	62.8	65.9	65.1	64.0
C. glabrata	11.1	10.7	12.1	11.7	11.2	11.7	12.0
C. tropicalis	7.5	7.4	7.6	7.5	7.6	8.0	8.3
C. parapsilosis	6.9	6.6	7.3	6.7	5.6	5.9	5.4
C. krusei	2.5	2.6	2.7	2.3	2.4	2.5	2.6
C. guilliermondii	0.7	1.0	0.8	0.7	0.7	0.5	0.5
C. lusitaniae	0.6	0.5	0.6	0.6	0.6	0.7	0.7
C. kefyr	0.3	0.4	0.5	0.5	0.6	0.5	0.6
C. inconspicua	0.1	0.2	0.3	0.3	0.3	0.3	0.4
C. famata	0.2	0.4	0.3	0.4	0.3	0.2	0.3
C. rugosa	0.7	0.6	0.4	0.2	0.1	0.1	0.2
C. dubliniensis	<0.1	0.1	<0.1	0.1	0.2	0.2	0.2
C. norvegensis	0.1	<0.1	0.1	0.1	0.1	0.2	0.1
C. lipolytica	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C. sake	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C. pelliculosa	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C. apicola						0.2	<0.1
C. zeylanoides	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
C. valida			<0.1	<0.1	<0.1	<0.1	<0.1
C. intermedia		<0.1		<0.1	<0.1	<0.1	<0.1
C. pulcherrima		<0.1	<0.1		<0.1	<0.1	<0.1
C. haemuloniae		<0.1	<0.1	<0.1	<0.1	<0.1	
C. stellatoidea							<0.1
C. utilis						<0.1	<0.1
C. humicola				<0.1		<0.1	<0.1
C. lambica							
C. ciferrii						<0.1	<0.1
C. colliculosa						<0.1	<0.1
C. holmii							<0.1
C. marina							<0.1
C. sphaerica							<0.1
Candida sp. NOS[c]	3.3	7.8	4.8	5.8	4.2	3.5	4.3

[a]Data compiled from reference 229.
[b]Includes all specimen types and all locations in hospitals from 142 institutions in 41 countries.
[c]*Candida* spp. NOS, *Candida* species not otherwise identified.

TABLE 9 Species distribution of *Candida* BSI isolates by clinical service[a]

Species	% Isolates by species and clinical service (*n*)[b]								
	GMED (1,339)	HEME (197)	SCT (58)	HIV (41)	NICU (26)	SOT (166)	ST (351)	SURG (662)	Total (2,019)
C. albicans	46.3	27.4	22.4	43.9	69.2	39.2	47.6	47.9	45.6
C. glabrata	26.6	25.9	32.8	29.3	0.0	38.6	26.8	24.0	26.0
C. parapsilosis	15.7	11.7	15.5	9.8	26.9	12.0	12.8	17.7	15.7
C. tropicalis	7.5	17.3	8.6	7.3	0.0	6.0	7.4	7.3	8.1
C. krusei	1.9	13.7	15.5	4.9	0.0	1.8	2.6	1.4	2.5
Other[c]	2.0	4.0	5.2	4.8	3.9	2.4	2.8	1.7	2.1

[a]Data compiled from references 94 and 227.
[b]GMED, general medicine; HEME, hematologic malignancy; SCT, stem cell transplant; HIV, human immunodeficiency virus/ AIDS; SOT, solid-organ transplant; ST, solid tumor; SURG, surgical (nontransplant).
[c]Other: 17 cases of *C. lusitaniae*, 5 of *C. guilliermondii*, 7 of *C. dubliniensis*, and 14 unknown *Candida* spp.

recovered from clinical material and generally is responsible for 90 to 100% of mucosal infections and for 40 to 70% of episodes of candidemia and IC (Table 8), although this may vary considerably according to the clinical service on which the patient is hospitalized (Table 9) (82, 83, 94, 185, 199, 218, 227, 283, 300).

Although approximately 95 to 97% of all *Candida*-associated IFI are caused by five species—*C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei*—the list of reported species continues to grow as laboratories are pushed to provide an identification to the species level as an aid in optimizing therapy of candidal infections (Table 8) (186, 187, 210, 211, 244, 272). Recently, our group reported the results of the ARTEMIS DISK Antifungal Surveillance Program in which species identification and the antifungal susceptibility were determined for 256,882 consecutive clinical isolates of *Candida* collected from cases of IC (e.g., isolates from blood, normally sterile sites, urine, and genital specimens) in 142 medical centers in 41 countries over a 10.5-year period (1997 through 2007) (229). As can be seen in Table 8 for the most recent years, 2001 through 2007, the list of species isolated from clinical specimens continues to grow each year and now totals 31 different species. This is most likely due to the fact that clinical laboratories increasingly are applying commercially available and classical mycological as well as molecular identification methods for species identification of isolates from clinical specimens (87, 88, 103). However, one cannot discount the possibility that given the increased numbers of immunocompromised individuals worldwide, an ever-increasing number of "nonpathogenic" species are truly emerging as opportunistic pathogens (14, 19, 36, 60, 104, 173, 199, 214–216, 218, 245, 272).

In addition to the increasing numbers of species reported in various surveillance programs, both temporal and geographic changes in species distribution may also be seen (Tables 8, 10, and 11). Despite the fact that *C. albicans* remains the most common species causing IC worldwide (Table 8), decreased isolation has been noted over time in several different geographic locations (Tables 10 and 11). Data from the various surveillance programs also illustrate that although the five most common species of *Candida* are the same worldwide, their distribution varies according to the clinical service (Table 9), from region to region (Table 10), and within regions and countries (Table 11).

Beyond the top five species, the remaining 3 to 5% of *Candida*-associated IFI are caused by 20 to 25 different species (Table 8), including *C. guilliermondii*, *C. lusitaniae*, *C. rugosa*, and a number of newly described cryptic species such as *C. dubliniensis*, *C. metapsilosis*, *C. orthopsilosis*, *C. fermentati*, *C. nivariensis*, and *C. bracarensis* (86, 129–131, 210, 214,

TABLE 10 Distribution of *Candida* bloodstream isolates by region, from the ARTEMIS global surveillance program, 2004 to 2007[a]

Species	% of total by region (*n*)				
	North America (2,116)	Latin America (1,348)	Europe (2,151)	Asia-Pacific (1,064)	Total (7,191)
C. albicans	51.8	46.0	58.5	49.1	52.7
C. glabrata	20.3	6.8	14.8	12.1	14.2
C. parapsilosis	14.4	18.5	9.8	13.8	13.9
C. tropicalis	8.5	18.5	8.5	17.3	11.8
C. krusei	1.9	4.5	4.7	2.5	3.3
C. lusitaniae	1.6	0.5	1.4	0.9	1.2
C. guilliermondii	0.4	3.3	0.6	1.2	1.1
C. kefyr	0.5	0.7	1.1	1.1	0.8
C. famata	0.2	0.6	0.2	0.7	0.4
C. pelliculosa	0.1	0.3	0.1	0.4	0.2

[a]Data compiled from reference 60a.

TABLE 11 Geographic and temporal variations in species distribution among bloodstream isolates of *Candida*

Location	Study period	Reference[a]	No. of isolates	% of total by species						
				C. albicans	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*	*C. guilliermondii*	*C. lusitaniae*
North America	2001–2004	218	2,773	51	22	14	7	2	<1	<1
North America	2001–2006	220	1,489	50	24	14	8	2		
North America	2001–2007	229	11,682	49	21	14	7	3	<1	<1
North America	2004–2006	60a	1,657	52	23	14	7	1	<1	2
North America	2004–2008	94	2,019	46	26	16	8	3		
United States	1992–1993	105	837	52	12	21	10	4		
United States	1995–1997	185	1,593	46	20	14	12	2	<1	1
United States	1995–1998	206	934	53	20	10	12	3		
United States	1998–2000	83	935	45	24	13	12	2		1.6
United States	2008	132	571	38	28	21	9			
Canada	1992–1994	302	415	69	8	10	7	1	<1	1
Canada	1999–2004	118	209	51	22	6	6	5	<1	<1
Europe	1997–1999	282	2,089	56	14	13	7	2	1	1
Iceland	1980–1999	12	172	64	12	10	6	<1		<1
Norway	1991–2003	256	1,415	70	13	6	7	2	<1	<1
France	1995	246	156	53	11	16	10	4		
Finland	1995–1999	232	479	70	9	5	3	8		
Spain	2002–2003	7	351	51	9	23	10	4		
Spain	2001–2006	52	1,997	47	12	19	10	5	3	1
Belgium	2002	276	211	55	22	13				
Portugal	2004	175	117	35	8	26	13			
Germany	2004–2005	30	561	59	19	8	8	1	<1	<1
Denmark	2003–2004	9	307	63	20	4	4	3	<1	<1
Denmark	2004–2006	10	1,089	60	21	4	3	4		
Scotland	2005–2006	174	241	52	23	12	2	1	3	2
Greece	1997–2007	61	135	33	8	13	7	14	2	13
E, W, NI[b]	2005–2008	96	7,429	53	19	11	4	1	1	1
Asia-Pacific	2001–2004	217	1,344	56	10	16	14	2	<1	<1
Asia-Pacific	2004–2007	60a	1,064	49	12	14	17	3	1	1
Australia	2001–2004	27	1,005	47	15	14	4			
South Korea	2002–2004	307	612	51	9	14	21	1	1	1
Korea	2004–2005	121	143	49	11	22	14	1	2	
Singapore	2004–2006	277	279	37	16	14	27	1	<1	
Taiwan	2006	250	145	57	14	6	22	<1		
Latin America	2001–2004	217	1,565	50	7	16	20	2	4	<1
Latin America	2004–2007	60a	1,348	46	7	19	19	5	3	<1
Latin America	1999–2000	78	103	42	8	21	24		3	2
Brazil	1995–2003	53	1,000	40	4	24	24	1	3	
Brazil	2003–2004	49	712	41	5	21	21	1	2	
Mexico	2004–2007	80	398	32	8	38	15	3	1	
Venezuela	2006–2007	181	145	28	15	27	20	3		1
Africa/Middle East	2001–2007	229	8,259	67	9	6	7	2	<1	
Kuwait	1996–2005	159	607	40	6	31	12	2		

[a]All studies cited were multicenter surveys.
[b]E, W, NI, England, Wales, and Northern Ireland.

215, 229, 275). Although these species must be considered to be rare causes of candidiasis, several have been observed to occur in nosocomial clusters or to exhibit innate or acquired resistance to one or more antifungal agents (47, 57, 60, 62, 86, 104, 173, 269).

Candida albicans

It is clear that among the various species capable of causing IC, *C. albicans* predominates (Table 8); however, the frequency with which this and other species are recovered from blood samples varies according to the age of the patients and the local, regional, or global setting (Tables 9 to 12) (1, 8, 49, 63, 82, 94, 105, 161, 207–209, 219, 267, 271, 283, 302). Globally, a decreasing trend in the rate of *C. albicans* isolation (7 to 10% decrease) was noted over an 8.5-year period (1997 to 2005) (219); however, this decrease appears to have stabilized in the last few years (Table 8) (229). In the United States, the incidence of *C. albicans* BSI was found to have decreased in both the Atlanta, GA, and Baltimore, MD, metropolitan areas between 1992 to 1993 and 2008 to 2009 (3, 132). *C. albicans* accounted for 45.6% of *Candida* BSI in a recent (2004 to 2008) U.S. multicenter survey, ranging from only 22.4% of *Candida* infections among stem cell transplant recipients to 69.2% of infections in the NICU (Table 9) (94). *C. albicans* represents a smaller proportion of BSI with increasing patient age (Table 12) (58, 94, 105, 206, 207, 211, 218), after exposure to azole antifungal agents (Table 13), (1, 79, 82, 92, 119, 148, 195), and in the ICU setting (283). Recently, Hachem et al. (82) found that in the setting of a large cancer referral center (M. D. Anderson Cancer Center, Houston, TX), factors that were predictive of *C. albicans* candidemia were an absence

TABLE 12 *Candida* species distribution in adults and children as reported by different candidemia surveillance programs[a]

Study population	Surveillance program and country[b]	Years	Reference	% of total				
				C. albicans	*C. glabrata*	*C. parapsilosis*	*C. tropicalis*	*C. krusei*
Adults	FDR, Canada	1992–1994	302	71	9	8	7	1
	NEMIS, United States	1993–1995	206	48	24	5	19	0
	MSG, United States	1995–1997	185	45	21	12	12	2
	NNIS, United States	1989–1999	206	59	12	10	11	NA[c]
	CDC, United States	1998–2000	83	44	26	13	13	NA
	CHR, Canada	1999–2004	118	49	25	5	6	5
	Sentry, global	1997–2000	206	50	23	12	10	2
	Norway	1991–2003	256	68	15	5	7	NA
	ECMM, Europe	1997–1999	282	58	16	10	8	NA
	ACS, Australia	2001–2004	27	49	17	15	5	5
	Demark	2004–2006	10	59	21	3	5	4
	Mexico	2004–2007	80	40	19	21	12	4
	Venezuela	2006–2007	181	30	22	14	26	6
Children	CDC, United States	1992–1993	105	53	0	45	0	0
	FDR, Canada	1992–1994	302	56	2	31	6	0
	NEMIS, United States	1993–1995	206	63	6	29	0	0
	MSG, United States	1995–1997	185	49	6	34	8	0
	NNIS, United States	1995–2004	70	58	2	34	4	<1
	CDC, United States	1998–2000	83	55	7	19	7	NA
	CHR, Canada	1999–2004	118	66	3	16	3	6
	Sentry, global	1997–2000	206	60	3	24	7	0
	Norway	1991–2003	256	82	<1	11	1	NA
	ECMM, Europe	1997–1999	282	54	3	30	4	NA
	ACS, Australia	2001–2004	27	43	4	39	2	1
	BCP, Spain	2002–2003	7	34	3	59	3	0
	Denmark	2004–2006	10	75	0	16	0	0
	Mexico	2004–2007	80	27	1	49	16	2
	Venezuela	2006–2007	181	25	7	41	13	0
	PATH, United States	2004–2008	94	69	0	27	0	0

[a]All studies were multicenter studies.

[b]FDR, Fungal Disease Registry; NEMIS, National Epidemiology of Mycoses Study; MSG, Mycoses Study Group; NNIS, National Nosocomial Infection Surveillance System; CHR, Calgary Health Region; ECMM, European Confederation of Medical Mycology; ACS, Australian Candidemia Study; BCP, Barcelona Candidemia Project; PATH, Prospective Antifungal Therapy.

[c]NA, not available.

TABLE 13 Multiple logistic regression analysis of independent risk factors predisposing patients to candidemia caused by different species[a]

Species	Risk factor	Odds ratio	95% Confidence interval
C. albicans	No neutropenia	1.5	1.2–2.4
	No fluconazole prophylaxis	3.3	2.0–5.6
	Solid tumor	2.5	1.5–4.0
C. tropicalis	Neutropenia	2.3	1.3–4.2
C. glabrata	Fluconazole prophylaxis	2.0	1.4–3.1
C. krusei	Fluconazole prophylaxis	5.3	2.9–9.5
	Neutropenia	5.4	2.7–10.7
C. parapsilosis	Catheter-related candidemia	2.5	1.6–3.8

[a]Data compiled from references 82 and 227.

of neutropenia, the presence of an underlying solid tumor, and a lack of prior use of fluconazole prophylaxis (Table 13). Whereas *C. albicans* accounted for 45% of *Candida* BSI in patients with solid tumors, it was only observed in 14% of patients with hematologic malignancies (82). These single-institution findings are supported by those of the multicenter survey reported by Horn et al. (94), where *C. albicans* was detected in 47.6% of *Candida* BSI in patients with solid tumors versus only 27.4% in those with hematologic malignancies (Table 9). Chow et al. (40) found the proportion of candidemia due to *C. albicans* to be increased among ICU patients without prior fluconazole exposure; however, this was not supported by Shorr et al. (265), who found that among patients whose BSI was diagnosed when they were in an ICU, no variable reliably differentiated infection with *C. albicans* from that with non-*C. albicans* species of *Candida*. These disparate findings emphasize the need to recognize that experiences in various hospital settings may differ greatly with regard to the epidemiology of candidemia (247, 267). It is essential to have knowledge of local epidemiological trends in order to guide initial therapy for *Candida* BSI (110, 187, 228, 247). Although *C. albicans* is usually considered to be an endogenous pathogen (i.e., infection arises from the patient's own flora), exogenous transmission may occur from patient to patient via the hands of health care personnel (13, 24, 145).

Candida glabrata

C. glabrata has emerged as an important and potentially antifungal-resistant fungal pathogen (1, 5, 8, 58, 63, 82, 83, 94, 105, 108, 109, 137, 142, 148, 173, 180, 210, 211, 218, 225, 228, 283). Trick et al. (283) have demonstrated that among the *Candida* species, *C. glabrata* alone has increased as a cause of BSI in U.S. ICUs since 1993. Most recently the species-specific incidence of candidemia due to *C. glabrata* in the Atlanta, GA, metropolitan area was shown to have increased from 1 infection per 100,000 population per year in 1992 to 1993 to 4.5 infections per 100,000 per year in 2008 to 2009 (3). The emergence of *C. glabrata* as an agent of IFI may be associated with the increased use of fluconazole and the decreased susceptibility of this species to this antifungal agent (82, 83, 269, 283). Suboptimal fluconazole dosing practices (low dose [<400 mg/day], short duration of treatment, and poor indications) may lead to an increased frequency of isolation of *C. glabrata* as an etiological agent of candidemia in hospitalized patients (10, 40, 75, 82, 94, 98, 109, 137, 180, 191, 264) and to increased fluconazole (and other azole) resistance secondary to induction of *Candida* drug resistance (CDR) efflux pumps (5, 20, 32, 33, 120, 137, 147, 180, 239, 258, 259).

On a global scale, the proportions of candidemia caused by *C. glabrata* vary from 22 to 26% in North America to 4 to 6% in Latin America (Tables 10 and 11). The frequency of isolation may vary considerably within each of the four geographic regions (Table 10), ranging from 2% (Indonesia) to 35% (Australia) in the Asia-Pacific region, from 3% (Turkey) to 28% (Germany) in Europe, and from 3% (Mexico) to 11% (Brazil) in Latin America (228).

Within the United States, the proportion of fungemia due to *C. glabrata* has been shown to vary from 11 to 37% across the nine U.S. Bureau of the Census Regions (208, 211, 225) and from <10 to >30% within single institutions over the course of several years (15, 142). In a recent U.S. study our group found that over the period from 2001 through 2007, *C. glabrata* accounted for 25% of 2,536 *Candida* BSI and was the second most common species isolated (Table 14) (225). The frequency of *C. glabrata* from 2001 to 2007 as a cause of candidemia in the United States ranged from 19% in the northeast to 34% in the West. By comparison with a previous study from our group encompassing the years 1992 to 2001 (208), the proportion of *Candida* BSI isolates that were *C. glabrata* increased in three of the four regions (West, Midwest, and South) and decreased only slightly in the northeast (from 21 to 19%) (Table 14). Notably, the rates of fluconazole resistance among the *C. glabrata* isolates from 2001 to 2007 increased compared to those from 1992 to 2001 in all regions except for the South, where the rate was unchanged (Table 14). Furthermore, the region with the highest prevalence of *C. glabrata* (West; 34%) had the lowest frequency of resistance (10%). Overall, 14% of the 2001 to 2007 U.S. *C. glabrata* isolates were resistant to fluconazole, compared with only 9% in the period from 1992 to 2001. In addition to the data shown in Table 14, review of fungal surveillance programs conducted in North America from 1992 to the present shows that the proportion of *Candida* BSI due to *C. glabrata* has increased significantly, from 8 to 12% in 1992 to 24 to 28% in the period from 2004 to 2008 (Table 11).

The variation in frequency of *C. glabrata* as a cause of BSI across clinical services has been shown by Horn et al. (94) (Table 9) and by Hachem et al. (82). Horn et al. (94) found that patients with *C. glabrata* fungemia were more likely than other patients with candidemia to be older and to have received a solid-organ transplant, whereas Hachem et al. (82) found that antifungal prophylaxis with fluconazole was a predisposing risk factor for *C. glabrata* fungemia among cancer patients (Table 13). In a recent study of candidemia in hematologic malignancy and stem cell transplant patients, Sipsas et al. (267) noted that whereas the proportion of fungemias due to *C. glabrata* increased from 12 to 31% during the era of fluconazole prophylaxis (1993 to 2002), it decreased to 5% in recent years (2001 to 2007), when echinocandins and voriconazole replaced fluconazole and itraconazole as prophylaxis agents in these patient populations (Table 15).

Numerous studies have shown that both colonization and infection with *C. glabrata* are rare among infants and children and increase significantly with patient age (Tables

TABLE 14 Temporal and geographic trends in the frequency of isolation and fluconazole resistance among BSI isolates of *C. glabrata* in the United States[a]

Region	Study period	Total no. of *Candida* BSI isolates	% of *C. glabrata* isolates	
			Among all isolates	Resistant to fluconazole
West	1992–2001	700	17	7
	2001–2007	61	34	10
Midwest	1992–2001	678	23	7
	2001–2007	1,420	28	12
Northeast	1992–2001	819	21	11
	2001–2007	897	19	17
South	1992–2001	1,486	15	11
	2001–2007	619	21	11
Total	1992–2001	3,683	18	9
	2001–2007	2,536	25	14

[a]Data compiled from references 208 and 225.

12 and 16). Importantly, more than one-third of *Candida*-associated BSI among patients >60 years of age are due to *C. glabrata* (Table 16). In a recent U.S. survey, our group found that the oldest age group (≥80 years) had the highest proportion of BSI isolates that were *C. glabrata* (Table 16) (225). Important new findings in this survey include an apparent increase in fluconazole resistance among *C. glabrata* BSI isolates from pediatric and adolescent patients (7% in the period from 1992 to 2001 and 27% in the period from 2001 to 2007) as well as a very low rate of fluconazole resistance (6.8%) among BSI isolates from older (≥70 years) patients (Table 17). The increased resistance seen in the younger patients reflects the increased use of fluconazole prophylaxis and treatment in this age group in recent years (21, 107, 143, 186, 187), whereas the low level of resistance in the oldest age groups may reflect the fact that although older individuals may have more frequent contact with the health care environment, they are less likely than younger individuals to undergo HSCT or solid-organ transplantation and thus less likely to receive fluconazole prophylaxis (106). Furthermore, it is now apparent that colonization with *C. glabrata* is much more common among older individuals irrespective of exposure to the health care environment (89, 126, 128, 238, 239). Such colonization may reflect a change in the ecology of *Candida* colonization with age rather than selection by drug pressure (126, 128, 236). Such colonizing strains are more likely to be fluconazole naïve and thus less likely to have acquired resistance to fluconazole (218).

TABLE 15 *Candida* species isolated from hematologic malignancy or HSCT patients with candidemia over three consecutive periods at M. D. Anderson Cancer Center[a]

Species	% of total (*n*) by species and study period		
	1988–1993 (230)	1993–2002 (281)	2001–2007 (173)
C. albicans	34	13	24
C. glabrata	12	31	5
C. krusei	7	24	17
C. parapsilosis	14	14	24
C. tropicalis	23	10	21
C. guilliermondii	1	1	2
C. lusitaniae	1	1	1

[a]Data compiled from reference 267.

The dramatic variation in the incidence of *C. glabrata* fungemia appears to be multifactorial (10, 82, 142, 208, 209, 225, 228, 247). Clearly, the frequency of isolation of *C. glabrata* from clinical specimens and decreased susceptibility to fluconazole and voriconazole may be driven by the use of fluconazole in the hospital setting (5, 82, 109, 120, 137, 180, 191, 247, 249). However, this statement is confounded by reports from outside the United States, including France (246), Italy (135, 281), Switzerland (144), Finland (232), Iceland (12), Taiwan (38, 39, 97, 98), and Norway (256) that indicate that *C. glabrata* has not increased as a cause of IC to the extent seen in the United States despite an increase in the use of fluconazole in each of those countries.

Despite the broad use of fluconazole worldwide, unfortunately it has been shown to be the antifungal agent that is most likely to be used inappropriately (either wrong dose or resistant organism) (74, 75, 109, 189). Garey et al. (75) have shown that 78% of patients with *C. glabrata* BSI were treated with a dose of fluconazole less than that recommended by the Infectious Disease Society of America (recommended dose, 12 mg/kg/day). Likewise, Klevay et al. (109) found that in contrast to patients with *C. albicans* BSI, those with *C. glabrata* infection were less likely to receive an adequate dose of fluconazole as empirical therapy (12% versus 52%; $P < 0.05$) and that time to receipt of adequate therapy was longer for patients infected with *C. glabrata* than for those infected with *C. albicans* ($P < 0.001$). Although Wilson et al. (296) found that fluconazole was a viable therapy for *C. glabrata* fungemia, they noted that higher doses of fluconazole (>400 mg/day) were more likely to achieve fungemia eradication than lower doses (≤400 mg/day) among patients who received only fluconazole (91% versus 50%, respectively [$P = 0.04$]). Finally, Sendid et al. (264) found that the emergence of *C. glabrata* as a cause of BSI in a French university hospital was linked to low-dose

TABLE 16 *C. glabrata* as a cause of BSI increases with patient age

Reference	% Distribution of *C. glabrata* by patient age group (yrs)									
	<1	1–9	10–19	20–29	30–39	40–49	50–59	60–69	70–79	≥80
225	5	4	9	20	17	22	30	29	29	32
181	7	0	11	13	21	25	20	24	NA[a]	NA
256	0	0	3	5	7	9	12	11	16	31
282	3	3	3	NA	NA	NA	NA	NA	19	NA
10	0	0	0	NA	NA	NA	NA	NA	NA	34
142	3	NA	NA	NA	NA	NA	NA	36	22	NA

[a]NA, data not available.

(50 to 100 mg/day) fluconazole usage, whereas the prevalence of *C. glabrata* decreased with an institutional shift to higher doses (>200 mg/day) of fluconazole concomitant with the introduction of voriconazole and caspofungin. The latter experience is very similar to that reported by Sipsas et al. (267) in a U.S. cancer center. Thus, it is now apparent that the dramatic variation in the frequency of *C. glabrata* as a cause of IC and its associated resistance profile may be influenced not only by exposure to azoles but also by the azole dosing regimen, the use of newer antifungal agents with greater potency against *C. glabrata*, patient age, underlying disease, and geographic location (5, 10, 40, 82, 109, 118, 120, 137, 142, 191, 225, 228, 247, 264, 267).

Candida parapsilosis

C. parapsilosis is the third most common species of *Candida* recovered from blood cultures in North America, accounting for 10 to 20% of *Candida* BSI (Tables 9 to 11) (41, 60a, 83, 94, 105, 118, 185, 207, 217, 220, 221, 229, 252, 284). In contrast to the situation in North America, in other countries and/or regions *C. parapsilosis*, not *C. glabrata*, is the most common non-*C. albicans Candida* species causing BSI (Tables 10 and 11). *C. parapsilosis* in an exogenous pathogen that may be found on skin rather than mucosal surfaces (7, 29, 41, 114, 123, 273, 284, 287). *C. parapsilosis* is known for the propensity to form biofilms on catheters and other implanted devices (41, 48, 114, 123, 284), for nosocomial spread by hand carriage, and for persistence in the hospital environment (7, 69, 123, 199, 241, 252, 260, 262, 284, 287). It is also well known for causing infections in infants and neonates (Tables 9 and 12) (70, 114, 124, 134, 221, 241, 253, 262, 263, 284, 287, 309). *C. parapsilosis* affects critically ill neonates and ICU patients likely because of its association with parenteral nutrition and central venous catheters (41, 82, 94, 114, 123, 262). Horn et al. (94) found that patients with *C. parapsilosis* BSI were more likely than other candidemic patients to have undergone recent surgery and to have a peripherally inserted central venous catheter, whereas Hachem et al. (82) reported that *C. parapsilosis* was the most common non-*C. albicans* species of *Candida* among solid-tumor patients and that catheter-related infection was an independent risk factor for *C. parapsilosis* BSI (Table 13). Notably, a recent report by Kabbara et al. (104) describes breakthrough *C. parapsilosis* BSI in HSCT patients receiving long-term caspofungin therapy, and Forrest et al. (67) found a strong correlation between caspofungin usage and a 400% increase in cases of *C. parapsilosis* BSI. Likewise, Sipsas et al. (267) reported that *C. parapsilosis* was isolated in 53% of hematologic malignancy and HSCT patients who developed candidemia while receiving caspofungin therapy. These authors suggested that the combination of central venous catheters and selection pressure (due to the frequent use of echinocandins) in the population may have resulted in the rise of *C. parapsilosis*, a species noted for decreased susceptibility to echinocandins (226) as well as the ability to adhere to the surface of catheters and thus cause candidemia (123, 237). In addition to these reports of an increasing frequency of *C. parapsilosis*, recent data from a population-based surveillance indicated that the species-specific incidence of *C. parapsilosis* BSI doubled in both the Atlanta, GA, and Baltimore, MD, metropolitan areas between 1992 to 1993 and 2008 to 2009 (3). Fortunately, BSI due to this species is associated with a significantly lower mortality rate than are infections due to the common species of *Candida* (1, 7, 83, 94, 168, 185, 260, 267, 284).

TABLE 17 Frequency of isolation and fluconazole resistance of BSI isolates of *C. glabrata* by patient age group, United States, 2001 to 2007[a]

Patient age group (yrs)	Total no. of *Candida* BSI isolates (%[b])	No. of *C. glabrata* isolates tested (% of total[c])	% of *C. glabrata* isolates resistant to fluconazole
≤1–19	194 (7.6)	11 (5.7)	27.3
20–49	684 (27.0)	137 (20.0)	23.4
50–69	964 (38.0)	287 (29.8)	11.5
≥70	694 (27.4)	207 (29.8)	6.8
All ages	2,536 (100)	642 (25.0)	14.0

[a]Data compiled from reference 225.
[b]Percent of total BSI isolates.
[c]Percent of total BSI isolates from each age group.

Recently, it was noted that 38% of *C. parapsilosis* BSI were acquired outside of the hospital, consistent with the association of this species with intravascular catheters and parenteral nutrition and with the increase in the management at home of patients with indwelling catheters and various chronic diseases (7, 83, 110, 284). The detection of BSI with *C. parapsilosis* should raise a "red flag" regarding breaks in catheter care and infection control procedures, as it usually signals the exogenous introduction of the offending pathogen into an already compromised host (41, 123, 200). Given the exogenous origin of *C. parapsilosis*, BSI due to this species should be preventable by careful attention to good infection control techniques, including hand hygiene and appropriate catheter placement and care (41, 48, 59, 123). Although antifungal prophylaxis with fluconazole has proven effective in preventing candidemia due to *C. parapsilosis* in the neonatal setting (107, 187, 284), a recent example of the emergence of fluconazole resistance in a strain endemic to a Finnish NICU underscores the importance of good infection control practices, rather than antifungal prophylaxis, in preventing infection with *C. parapsilosis* (262).

Candida tropicalis

C. tropicalis has long been considered to be an important cause of IFI in patients with cancer, especially leukemia, and in HSCT recipients (1, 18, 82, 94, 111, 148, 298). Horn et al. (94) found *C. tropicalis* to be especially prominent among patients with hematologic malignancies (17.3% of *Candida* BSI) versus patients on other clinical services (0.0 to 8.6%) (Table 9), and Hachem et al. (82) reported that the presence of neutropenia was an independent risk factor for *C. tropicalis* fungemia in cancer patients (Table 13). Kontoyiannis et al. (111) have shown that cancer patients with *C. tropicalis* fungemia are more likely to have leukemia, neutropenia, prolonged fungemia, and more days in the ICU than are those with *C. albicans* fungemia. Among patients with neutropenia who are found to be colonized with *C. tropicalis*, as many as 60 to 80% eventually develop IC with this species (201, 257, 298). Consequently, *C. tropicalis* has been considered to exhibit increased virulence, especially in those individuals with disrupted mucosal integrity (111, 291, 298). Given these considerations, prophylactic therapy with fluconazole for patients with neutropenia has been used in an effort to decrease infections due to *C. tropicalis* as well as *C. albicans* (1, 7, 148). Indeed, Hachem et al. (82) have shown a significant decrease in the frequency of *C. tropicalis* BSI associated with the widespread use of fluconazole prophylaxis in patients with hematologic malignancies and HSCT during the 1990s and 2000s. *C. tropicalis* accounted for 23% of *Candida* BSI (second in rank order) in the pre-fluconazole era (1988 to 1992) versus only 9.6% (fifth in rank order) in the years following the introduction of fluconazole (1993 to 2002) (Table 15). Sipsas et al. (267) found that changes in antifungal prescribing patterns from the use of fluconazole and itraconazole (1993 to 2002) to caspofungin and voriconazole (2001 to 2007) were associated with an increase in the incidence of BSI due to *C. tropicalis* (as well as *C. parapsilosis*) (Table 15). Overall, *C. tropicalis* accounted for 10 to 12% of *Candida* BSI in North America during the 1990s and 7 to 8% in the 2000s (Table 11).

Despite a decreasing proportion of candidemia caused by *C. tropicalis* in North America, the global trend appears to be an increasing frequency of infection with this species, with an increased frequency of isolation ranging from 5.4% of IC episodes in the period from 1997 to 2000 to 8.0% in the period from 2005 to 2007 (229). Whereas *C. tropicalis* is only the fourth most common species of *Candida* causing BSI in North America (8.5% of BSI) (Table 10), it ranks second in both Latin America (18.5%) and the Asia-Pacific region (17.3%) (Table 10). The increased prominence of *C. tropicalis* in the Asia-Pacific region has been recognized in several recent surveys (37, 98, 121, 301, 303–307). Reports from Taiwan have also highlighted the emergence of fluconazole resistance in *C. tropicalis* from a variety of different specimen types (303, 306). These data are supported by the most recent ARTEMIS surveillance data showing that the highest rates of resistance among *C. tropicalis* isolates to both fluconazole and voriconazole were seen among isolates from the Asia-Pacific region (229).

Candida krusei

C. krusei (teleomorph, *Issatchenkia orientalis*) accounts for 1 to 5% of all *Candida*-associated BSI (Tables 8 and 10) and is best known for its propensity to emerge in settings where fluconazole is used for prophylaxis (1, 7, 82, 92, 94, 148, 222, 298). The prevalence of *C. krusei* appears to be highest in Eastern Europe, namely Hungary, Russia, and the Czech Republic (229). Whereas these three countries accounted for 21% of the *Candida* isolates in the ARTEMIS surveillance program, they accounted for 38% of all *C. krusei* isolates (229).

Similar to *C. tropicalis* infections, *C. krusei* infections occur most often in patients with neutropenia, and colonization of patients is often predictive of BSI with this species (1, 7, 82, 92, 94, 201, 257, 298). Both neutropenia and prophylaxis with fluconazole were independent risk factors for *C. krusei* BSI at the M. D. Anderson Cancer Center (Table 13), where *C. krusei* increased as a cause of *Candida* BSI from 7.4% of infections (fifth in rank order) in the pre-fluconazole era (1988 to 1992) to 24.2% of infections (second in rank order) following the introduction of fluconazole (1993 to 2002) (Table 13) (82). Notably, *C. krusei* dropped to fourth in rank order (17% of *Candida* BSI), commensurate with a decline in fluconazole and itraconazole usage in favor of echinocandins and voriconazole as prophylactic agents at this institution (Table 13) (267).

Horn et al. (94) found *C. krusei* to be associated most commonly with prior use of antifungal agents, hematologic malignancy, neutropenia, and receipt of HSCT (Table 9). *C. krusei* is best known for resistance to fluconazole; however, it may also exhibit decreased susceptibility to amphotericin B and flucytosine, further complicating therapy (187, 218, 222, 272). Fortunately, *C. krusei* expresses a fluconazole-resistant, voriconazole-susceptible phenotype (222, 229). Studies have demonstrated that the voriconazole activity against this species may be attributed to the enhanced binding of this triazole to the target enzyme compared to that of fluconazole (72). BSI due to *C. krusei* clearly has emerged among those blood and marrow transplant recipients receiving fluconazole prophylaxis (1, 82, 148, 299), but fluconazole exposure alone cannot explain the reported increase in infections caused by this species, since an increase in prevalence of *C. krusei* predated the use of fluconazole in some institutions (18, 100, 155, 298). Whereas Hachem et al. (82) found that infections caused by *C. krusei* were strongly associated with fluconazole prophylaxis and neutropenia at the M. D. Anderson Cancer Center (Table 13), Lin et al. (127) found this not to be the case in the less-specialized tertiary care setting of the University of Chicago Hospitals. Lin et al. (127) found that patient exposure to

piperacillin-tazobactam and vancomycin was more important than exposure to fluconazole in promoting *C. krusei* BSI. They suggested that these antibacterial agents may promote skin and GI tract colonization with *C. krusei* by altering the normal flora and thereby decreasing the colonization resistance of the host (Table 1) (127).

Other *Candida* Species

Among the remaining 20 to 25 species of *Candida* that may cause IC (Table 8) (229), there are several that merit discussion either because they have been shown to cause clusters of infection in the hospital setting, because they appear to be increasing in frequency, or because they exhibit decreased susceptibility to one or more antifungal agents and therefore pose a threat of emergence in certain settings (14, 19, 36, 60, 64, 104, 173, 199, 210, 214, 215, 229, 245, 272). Those species addressed in this chapter include *C. lusitaniae*, *C. guilliermondii*, *C. rugosa*, *C. inconspicua*, and *C. norvegensis*.

C. lusitaniae (teleomorph, *Clavispora lusitaniae*) most often causes fungemia in patients with malignancies or other serious comorbid conditions (14, 86). Atkinson et al. (14) recently found that patients with *C. lusitaniae* fungemia were more likely than those with *C. albicans* fungemia to have neutropenia, to have stem cell transplantation, and to have received prior antifungals. *C. lusitaniae* is often mentioned in the literature as being capable of developing resistance to amphotericin B during the course of therapy and may manifest as breakthrough infection in immunocompromised patients on amphotericin B therapy (23, 86, 90, 150, 154, 156, 157, 169, 183, 254, 308). Atkinson et al. (14) found that in contrast to patients with *C. albicans* fungemia, patients with BSI due to *C. lusitaniae* had an increased treatment failure rate when they were treated with an amphotericin B-based regimen (10% versus 38%, respectively; $P = 0.028$), and a greater need for subsequent ICU admission (22% versus 54%, respectively; $P = 0.04$). *C. lusitaniae* appears to be unique among *Candida* species due to an acquired or inducible ability to exhibit high-frequency phenotypic switching from amphotericin B susceptibility to resistance upon exposure to the drug (14, 150, 156, 308). Atkinson et al. (14) demonstrated that resistance to amphotericin B may be easily selected out from originally amphotericin B-susceptible strains in vitro. Furthermore, they found that amphotericin B is considerably less fungicidal against *C. lusitaniae* than against *C. albicans* (14). These findings indicate that *C. lusitaniae* may be less amenable to therapy with amphotericin B irrespective of its initial in vitro susceptibility results (187).

C. guilliermondii and *C. rugosa* are uncommon species of *Candida* that appear to be increasing in frequency as causes of IC in some regions of the world (Table 10) (214, 215). Both species have been responsible for clusters of infection in the hospital setting, and both demonstrate decreased susceptibility to amphotericin B, fluconazole, and the echinocandins (46, 47, 57, 60, 62, 149, 210, 214, 215).

C. guilliermondii (teleomorph, *Pichia guilliermondii*) is known to be a normal component of the human skin and mucosal floras (152, 158) and is rarely associated with invasive infections such as endocarditis (286), pericarditis (288), osteomyelitis (280), and peritonitis and fungemia (77, 104, 146, 149, 190, 197, 215). Infection with this species is often catheter related and has been found to be more common among patients with cancer than among the general hospital population (77, 146). Others have noted catheter-related fungemia with *C. guilliermondii* among patients with prior cardiovascular or abdominal surgery (105, 149).

One of the initial descriptions of IC due to *C. guilliermondii* was that of a fatal case of disseminated infection in which the patient died despite amphotericin B therapy (57). The isolate was shown by in vitro testing to be resistant to amphotericin B. Subsequently, others have documented the failure of amphotericin B therapy in invasive or ocular infection with potential in vitro resistance (MICs, 1 to 4 μg/ml) or neutropenia (7, 39, 112). *C. guilliermondii* is known to show reduced susceptibility to the echinocandin class of antifungal agents (215, 220, 226), and recently, Kabbara et al. (104) reported breakthrough *C. guilliermondii* BSI in an HSCT recipient receiving caspofungin prophylaxis. The reduced susceptibility of this species to echinocandins may come into play when infections with *C. guilliermondii* involve anatomical sites where adequate free drug levels cannot be readily obtained (224, 226).

C. rugosa is an uncommon cause of fungemia (240); however, it has been implicated in clusters of nosocomial BSI in the United States and in Latin America (47, 62). Like *C. guilliermondii*, *C. rugosa* is reported to exhibit decreased susceptibilities to polyenes, azoles, and echinocandins and may cause catheter-related fungemia in seriously ill patients (60, 62, 173, 214, 240). In a multicenter survey of invasive candidiasis (214), our group found that *C. rugosa* was recovered most often in cultures of blood and urine obtained from patients hospitalized on medical and surgical inpatient services.

C. inconspicua and *C. norvegensis* are both similar phenotypically to *C. krusei* (138–141, 170, 274). Like *C. krusei*, they exhibit intrinsic resistance to fluconazole yet remain susceptible to voriconazole (229). Although quite uncommon in most regions of the world, these two species have been recognized for some time in Europe as fluconazole-resistant causes of candidal colonization and infection (17, 54, 64, 91, 139, 140, 171, 172, 255). *C. inconspicua* has been reported to be a cause of fungemia in human immunodeficiency virus-infected patients and in patients with hematologic malignancies (17, 54). The latter appeared to be due to a common source within the hospital environment, as all affected patients were infected with the same strains, based on DNA restriction profiles (54). *C. inconspicua* may be especially common in Hungary, where Majoros et al. (138–140) reported on 57 isolates from 48 patients. Recently, our group found that 75% of 575 isolates of *C. inconspicua* originated from Eastern Europe (Hungary, Russia, and the Czech Republic) during the 10.5-year (1997 to 2007) period of the ARTEMIS surveillance program (229). Most isolates (70%) were from respiratory tract samples, but wound, blood, and genital isolates were also obtained.

C. norvegensis has been reported from clinical specimens in Norway, The Netherlands, and Japan (274). In the ARTEMIS surveillance program, 83% of 248 isolates of *C. norvegensis* were from Europe and 31% of the isolates were from Eastern Europe, namely, Hungary, Russia, and the Czech Republic (229). Although most isolates to date have been from respiratory specimens (229, 255), our group has identified this species from blood (15% of 248 isolates), other normally sterile sites (17%), and urine (9%) (229).

Cryptic Species of *Candida*

As the methods of species identification have evolved from the more traditional phenotypic methods to nucleic acid-based techniques, previously unrecognized "cryptic" species of *Candida* have been recognized (103). These cryptic species include *C. dubliniensis*, previously indistinguishable from

C. albicans (275); *C. orthopsilosis* and *C.metapsilosis* in the *C. parapsilosis* complex (129, 279); *C. fermentati* in the *C. guilliermondii* complex (16, 130, 261); and *C. nivariensis* and *C. bracarensis* in the *C. glabrata* clade (22, 31, 131). The frequency of occurrence and clinical importance of these species are only now being investigated.

In contrast to the other cryptic species of *Candida*, *C. dubliniensis* can now be differentiated from *C. albicans* by nonmolecular methods (73). Although originally associated strictly with oropharyngeal candidiasis, *C. dubliniensis* is now recognized as an (uncommon) cause of BSI (44, 45, 153, 163). It was recently shown to rank 12th out of 15 species of *Candida* causing BSI in the ARTEMIS surveillance project (60a). Although fluconazole resistance was previously reported to be common among isolates of *C. dubliniensis* from cases of oropharyngeal candidiasis (44, 45), subsequent studies have found most isolates to be susceptible to fluconazole and the newer azoles as well as the echinocandins (60, 153, 205, 226).

C. orthopsilosis (formerly *C. parapsilosis* group II) and *C. metapsilosis* (formerly *C. parapsilosis* group III) are newly recognized members of the *C. parapsilosis* complex (279). Lockhart et al. (129) recently demonstrated that among 1,929 BSI isolates presumed to be *C. parapsilosis*, 91.3% were *C. parapsilosis*, 6.1% were *C. orthopsilosis*, and 1.8% were *C. metapsilosis*. Notably, the percentage of *C. parapsilosis* isolates that were confirmed to be *C. orthopsilosis* increased from 4.5% in the years 2001 to 2004 to 8.3% in the years 2005 and 2006 (129). *C. orthopsilosis* accounted for only 5% of *C. parapsilosis* complex isolates from North America, 59% of which were isolated from patients in the over-60-year age group (versus only 38% of *C. parapsilosis* isolates). *C. orthopsilosis* isolates constituted much higher proportions of *C. parapsilosis* complex isolates in Latin America (12.7%) and the Asia-Pacific region (11%) than in either Europe (3.9%) or the United States (5.1%) (129). Neither *C. orthopsilosis* nor *C. metapsilosis* is resistant to fluconazole or the echinocandins; however, decreased susceptibility to amphotericin B may occur (60). At present there are few clinical or epidemiological data concerning these two cryptic species. In addition to sharing several phenotypic features with *C. parapsilosis*, both *C. orthopsilosis* and *C. metapsilosis* have been shown to be capable of forming biofilms on biomedical substrates (117).

C. fermentati is a member of the *C. guilliermondii* complex, for which there is almost no clinical information (16, 116, 285). Lockhart et al. (130) recently found that among 149 *C. guilliermondii* complex isolates, 13 (8.7%) were further identified as *C. fermentati* using the molecular identification method of Lan and Xu (116). The *C. fermentati* isolates originated from North America, South America, Asia, and Australia. Previous *C. fermentati* isolates have been reported from India, the United Kingdom, Japan, and Brazil (152, 261, 285). Eight of the 11 isolates for which epidemiological data were available were BSI isolates, similar to the 78% of *C. guilliermondii* isolates that were from BSI (130). Whereas 80% of the patients with a *C. fermentati* infection were ≥40 years old, only 38% of the *C. guilliermondii* isolates came from patients older than 40 years of age. Only 10% of *C. fermentati* isolates were from the pediatric population, while 29% of *C. guilliermondii* isolates were from pediatric patients. The in vitro susceptibility of *C. fermentati* isolates to antifungal agents, including the azoles and echinocandins, were not significantly different from those for *C. guilliermondii*. At present, *C. fermentati* isolates are not sufficiently different from *C. guilliermondii* isolates in terms of antifungal susceptibility to warrant routine identification in the clinical microbiology laboratory.

C. nivariensis and *C. bracarensis* are two uncommon species that are described as phenotypic mimics of *C. glabrata* (4, 51). Recently, Borman et al. (31) reported the isolation of 16 strains of *C. nivariensis* from a variety of clinical specimens (including deep, usually sterile sites) at 12 different hospitals in the United Kingdom, suggesting that this species was much more widespread than originally appreciated when the species was first described (4). Furthermore, these isolates were shown to have decreased susceptibility to itraconazole, voriconazole, and fluconazole (31). Likewise, a fluconazole-resistant strain of *C. nivariensis* was obtained from the blood of a patient in Japan with catheter-related fungemia (71). In addition to molecular methods of identification, *C. nivariensis* may be differentiated from *C. glabrata* by growing as white, rather than pink or mauve, colonies on CHROMagar and by the inability to assimilate trehalose (31, 131).

Given the suggestion of Borman et al. (31) that *C. nivariensis* was an emerging pathogen with multidrug resistance, Lockhart et al. (131) examined a total of 1,598 isolates presumed to be *C. glabrata* from 98 medical centers in 28 countries using CHROMagar, PCR, and peptide nucleic acid fluorescence in situ hybridization to determine what proportion were *C. nivariensis* (and *C. bracarensis*) and whether these isolates indeed had high rates of resistance to antifungal agents.

Among the 1,598 presumed *C. glabrata* isolates, 1,592 (99.7%) were confirmed to be *C. glabrata*, 2 isolates (both from North America) were *C. bracarensis*, and 1 isolate (from Australia) was *C. nivariensis*. The two isolates of *C. bracarensis* were from sputum (isolated in 2002) and from blood (isolated in 2004), and the isolate of *C. nivariensis* was from pleural fluid. None of the 400 isolates from 32 medical centers in Europe were identified as either *C. nivariensis* or *C. bracarensis*. Despite the reports of almost uniform azole resistance in isolates of *C. nivariensis* (31, 71), the lone isolate of *C. nivariensis* from Australia was neither azole nor echinocandin resistant (131). One of the two isolates of *C. bracarensis* demonstrated decreased susceptibility to fluconazole (MIC, 16 mg/liter) and to amphotericin B (MIC, 8 mg/liter).

To date, the total number of published reports concerning either *C. bracarensis* or *C. nivariensis* is small. The first isolates of *C. nivariensis* were reported from the Canary Islands in 2005 from a pulmonary abscess, blood, and urine (4). Subsequently, single isolates have been reported associated with a catheter-related BSI in Japan (71) and as a cause of oropharyngeal candidiasis in Indonesia (290). Among the 16 isolates reported from the United Kingdom, 9 were from normally sterile sites, including blood, ascitic fluid, and peritoneal fluid (31).

Even less is known of *C. bracarensis*. Following the publication of one isolate from a case of vaginitis in Portugal and another from a blood culture in the United Kingdom in 2006 (51), only three more clinical isolates have been described, isolates from the throat and stool of two oncology patients and another from a pelvic abscess, all from Baltimore, MD (22).

The study of Lockhart et al. (131) suggests that *C. nivariensis* and *C. bracarensis* isolates make up only a very small percentage of the *C. glabrata* isolates from global surveillance. Other published data indicate that they may be more locally or regionally prevalent (22, 31). Colony color on CHROMagar may be a useful first step in differentiating

these two species from C. *glabrata*. Whereas not all *Candida* isolates growing as white colonies on CHROMagar are C. *nivariensis* or C. *bracarensis*, all isolates of these two species have been white on CHROMagar (22, 31, 131).

RISK FACTORS

The burden of IC is tremendous in terms of morbidity, mortality, and cost, and it is clear that we must do more than simply seek better therapeutic agents if we are to have an impact on this burden (59, 68, 74, 75, 110, 162, 164, 189, 266, 289, 293). *Candida* BSI have been shown to have some of the highest rates of inappropriate therapy and hospital mortality among all etiological agents examined (84, 99, 108, 162, 164, 189, 300). The most common causes of inappropriate therapy for *Candida* BSI are omission of initial empirical therapy (74, 108, 164, 189) followed by incorrect dosing of fluconazole (11, 75, 94, 189, 193). Such inadequate therapy has been linked directly to mortality (74, 108, 164, 189). Thus, despite an unprecedented array of new, potent, and nontoxic antifungal agents, we often fail in the management of these infections (59, 74, 75, 164, 189, 218, 235).

The lack of specific clinical findings and slow, insensitive diagnostic testing complicate the early recognition and treatment of IC (6, 164, 165, 178, 189, 265, 266, 289). Most authors recommend the use of clinical risk factors to identify patients who may benefit from prophylactic or empirical antifungal therapy in the proper clinical setting (35, 110, 165, 176, 177, 179, 182, 234, 243, 270, 293). Unfortunately, the predominant risk factors for IC are common iatrogenic and/or nosocomial conditions (Table 1) (179, 199). Additional meaningful stratification of identified risk factors will be required to identify those high-risk patients who would derive maximal benefit from early therapeutic interventions (110, 179, 266, 270, 293).

The risk factors associated with candidemia and IC have been well established and have not changed substantially in more than two decades (Table 1) (26, 59, 165, 176, 178, 216, 233, 235, 295). Those determined to be independent risk factors for IC on the basis of multivariate analysis include exposure to broad-spectrum antimicrobial agents, cancer chemotherapy, mucosal colonization by *Candida* spp., indwelling vascular catheter (especially central venous catheter), total parenteral nutrition, neutropenia, prior surgery (especially GI surgery), and renal failure and hemodialysis (Table 1) (26, 59, 122, 164, 176, 179, 182, 230, 270, 295).

It is important to understand that the risk for IC in the hospital is a continuum (59, 133, 216, 270, 293). Certain hospitalized individuals are clearly at increased risk of acquiring candidemia during hospitalization as a result of their underlying medical condition: patients with hematologic malignancies and/or neutropenia, those undergoing GI surgery, premature infants, and patients greater than 70 years of age (Table 1). Among patients with IC in the United States, the mean time to onset of candidemia was at 22 days of hospitalization (185, 300). Thus, it must be emphasized that nosocomial IC typically affects individuals with severe illnesses who have prolonged hospitalization (199).

Within the high-risk groups, specific additional exposures have been recognized to further increase the risk of IC: the presence of vascular catheters, exposure to broad-spectrum antimicrobial agents, renal failure, pancreatitis, mucosal colonization with *Candida* spp., prolonged (>7 days) ICU stay, mechanical ventilation, and receipt of total parenteral nutrition (Table 1) (26, 179, 293, 295). Compared to controls without the specific risk factors or exposures, the likelihood of these already high-risk patients contracting candidemia in the hospital is approximately 2 times greater for each class of antibiotics they receive, 7 times greater if they have a central venous catheter, 10 times greater if *Candida* has been found to be colonizing other anatomical sites, and 18 times greater if the patient has undergone hemodialysis (293, 295). Hospitalization in the ICU provides the opportunity for transmission of *Candida* among patients (13, 151) and has been shown to be an additional independent risk factor (26, 63, 177, 253, 293). When two or more of these risk factors are present, the probability of infection increases exponentially (165, 293). Thus, Wenzel and Gennings (293) have demonstrated that in a patient who had received eight different antimicrobials and had *Candida* cultured from a surgical wound and drain effluent, the risk of developing candidemia was 832 times that of a similar patient without antimicrobial therapy or *Candida* colonization.

Several investigations have now used these risk factors to develop clinical risk assessment strategies that could be used in the ICU to (i) predict certain rates of IC, (ii) identify a substantial proportion of patients who actually go on to develop IC, and (iii) be practical for use as selection tools for risk-targeted prevention (prophylaxis) or treatment (preemptive or empirical) strategies (122, 179, 293). Preliminary application of these strategies shows that risk stratification is possible and practical in the ICU; however, their clinical utility remains to be established in prospective studies (179).

Although it is reasonable to focus attention on IC in patients in the ICU, the emergence of early-onset/ CO candidemia cannot be ignored (110, 266). CO candidemia is clearly an HAI, and affected individuals share many of the risk factors associated with nosocomial candidiasis despite coming to medical attention from an outpatient setting (110, 266). This recently appreciated aspect of the spectrum of IC further complicates efforts to affect the mortality of IC and adds weight to the already pressing need for improved rapid diagnostics (110, 266).

MORTALITY, LOS, AND COST ASSOCIATED WITH CANDIDEMIA AND IC

The consequences of candidemia in hospitalized patients are severe. Patients with candidemia have been shown to be at a twofold-greater risk of death in hospital than are patients with noncandidal BSI (231). Shorr et al. (266) have extended this to include those patients with CO candidemia, for whom the mortality rate is approximately triple that of patients with CO gram-negative bacteremia (Table 7). In a multicenter U.S. study of candidemia, risk factors for mortality included an APACHE II score of >18 ($P < 0.001$), cancer ($P = 0.002$), the presence of a urinary catheter ($P = 0.004$), male sex ($P = 0.004$), the use of corticosteroids ($P < 0.001$), and the presence of an arterial catheter ($P < 0.001$) (185).

Estimates of the mortality attributable to candidemia and other forms of IC have been reported from retrospective matched-cohort studies conducted in single institutions (81, 160, 194, 235, 294) and in the context of population surveillance studies (Table 18) (85, 162, 298, 309). The weight of evidence provided by these studies suggests that candidemia or IC is associated with a substantial excess (attributable) mortality ranging from 10 to 49% (Table 18) (65). Furthermore, these data demonstrate that candidemia carries no less risk of death during hospitalization today than

TABLE 18 Incidence, excess mortality rates, LOS, and costs attributable to candidemia[a]

Study period	Location/patient population	No. cases/ 100,000/yr[b]	Mortality (%)	LOS (days)	Cost ($)	Reference
1983–1986	Iowa	NA	38.0	30.0[c]	NA	294
1996	Baltimore, MD, SICU	NA	19.0	17.0	21,590	194
1997–2001	Iowa	6.0	49.0	10.5	NA	81
1998–1999	Connecticut	7.1	19.0	3.4	6,214	162
1998–2000	Baltimore, MD	24.0	24.0	12.9	29,094	162
1998–2000	Baltimore, MD, and Connecticut, pediatrics	NA	10.6	9.0	28,500	268
2000	United States, pediatrics	43.0[d]	10.0	21.1	92,266	309
2000	United States, adults	30.0[d]	14.5	10.1	39,331	309
2000	St. Louis, MO	NA	35.7	NA	44,051	235
2007	Manchester, United Kingdom	NA	34.7	12.1[b]	13,120	85
2007	Chandigarah, India, pediatrics	NA	35.9	NA	NA	34
2009	North Carolina					
	Infants	NA	35.6	51.0	142,394	160
	Pediatrics	NA	28.7	36.9	133,871	
	Adults	NA	43.0	20.7	56,725	

[a]SICU, surgical ICU; NA, data not available.
[b]Cases per 100,000 population.
[c]LOS determined in surviving pairs only.
[d]Cases per 100,000 admissions.

it did 20 years ago (59, 81, 294), despite the introduction of new antifungal agents with superior safety, spectrum, and potency against most species of *Candida* (199, 272).

Treatment of candidemia is often found to be inadequate due to the delay in administration of therapy, treatment with an agent to which the organism is resistant, inadequate dose or duration of treatment, or the absence of any treatment at all (11, 14, 74, 75, 108, 162, 164, 189, 278). Several studies have now shown that delays in the initiation of adequate antifungal therapy of >12 h (164), >24 h (74, 76, 189, 278), and >48 h (25) were independently associated with mortality in candidemia patients (Table 19). Taur et al. (278) have shown that in cancer patients with candidemia, the time from blood culture collection to positivity (incubation period) is associated with in-hospital mortality: the mortality rate increased 1.025-fold for every additional hour of blood culture incubation, and a 24-h delay in blood culture positivity would nearly double the risk of death.

A population-based study of candidemia found that removal of vascular catheters, in addition to receipt of at least 5 days of antifungal treatment, was independently associated with a decreased risk for both early and late mortality (7). Likewise, Morgan et al. (162) demonstrated that the attributable mortality rate was lower among patients who received adequate (>7 days of a systemically active agent) treatment for candidemia (11% in Connecticut and 16% in the Baltimore, MD, metropolitan area) than among patients who did not receive adequate treatment (31% in Connecticut and 41% in Baltimore). Finally, Parkins et al. (189) found that empirical therapy with an antifungal agent to which the organism was susceptible in vitro was associated with a significant reduction in all-cause mortality, from 46 to 27% ($P = 0.02$). Notably, empirical fluconazole therapy was more likely to be deemed inadequate (due to both inadequate dosing and in vitro resistance), and inadequate therapy was an independent predictor of death in hospital (189). Thus, reduction of the mortality rate due to candidemia and IC is dependent upon the administration of appropriate antifungal therapy (right drug and dose) early in the course of infection and for an adequate duration.

Despite numerous barriers to improving outcomes in patients with candidemia and IC, the way forward may be to consider the guidelines presented in the Surviving Sepsis Campaign (56, 110, 266). Sepsis as a whole is estimated at more than 500,000 cases annually in the United States, with a reported mortality rate of 35 to 60% (43, 56, 248). Given that each hour of delay in the administration of antibiotics in sepsis is associated with an 8% increase in the mortality rate (115), the Surviving Sepsis Campaign developed evidence-based practice guidelines with the intent of standardizing the approach to sepsis in order to reduce mortality (56). The current international guidelines for the management of sepsis recommend (in addition to numerous supportive interventions) the initiation of empirical antimicrobial therapy within 1 h of presentation with severe sepsis (56). Thus far, studies have shown that the case-fatality rate can be reduced by 33 to 77% with early administration of appropriate antibiotics (coupled with other supportive interventions) (28, 125, 136). Given this level of success, coupled with the lack of progress with rapid fungal diagnostics, Kollef et al. (110) have suggested that perhaps patients presenting to the emergency department with sepsis and who have risk factors for an HAI may require early empirical therapy with an antifungal agent to reduce mortality. Given the changing patterns of candidemia with movement from the ICU to the outpatient setting and its disproportionate burden on the health care system, aggressive evaluation of patients at risk for candidemia irrespective of when or where they present to medical attention is warranted (110, 266).

TABLE 19 Delay in treatment of *Candida* BSI is a potential risk factor for hospital mortality

Reference	Patient stratum	No. of patients	% Mortality	
			With delay (h)[a]	Without delay
164	All	157	33.1 (>12)	11.1
	APACHE II score of ≤15	90	23.5 (>12)	0.0
	APACHE II score of >15	67	46.0 (>12)	25.0
74	All	230	23.7 (24)	15.4
			36.4 (48)	
			41.4 (≥72)	

[a]Values in parentheses are numbers of hours of delay in administering systemic antifungal therapy after the first positive blood culture was drawn.

Several studies have examined the excess LOS and hospital costs attributable to IFI due to *Candida* (Table 18). Candidemia patients have been shown to have between 3 and 30 more hospital days than uninfected patients with the same underlying disease and disease severity (Table 18). Given the prevalence of *Candida* infections and their attributable impact on mortality and LOS, it is not surprising that these infections are associated with substantial health care costs (68, 155a, 194, 199, 242, 297). The excess costs attributable to candidemia range from $6,214 to $142,394 per episode depending on geographic location and patient type (Table 18). It is estimated that 85% of the increase in cost of care for patients with candidemia is due to the excess LOS (242). Because each case of candidemia adds tens of thousands of dollars to hospitalization costs, the estimated health care cost associated with hematogenously disseminated candidiasis is $2 billion to $4 billion/year in the United States alone (155a, 199, 242, 297, 309).

ANTIFUNGAL SUSCEPTIBILITY

Among the 16 species of *Candida* discussed in this chapter, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. lusitaniae*, *C. dubliniensis*, *C. metapsilosis*, and *C. orthopsilosis* remain reliably susceptible to fluconazole and voriconazole (Table 20) (129, 229). In the ARTEMIS surveillance program, 90% of 201,653 isolates of *Candida* were susceptible to fluconazole and 95% of 197,619 isolates were susceptible to voriconazole (Table 20). Decreased susceptibility to fluconazole (<75% susceptible) was seen with *C. glabrata* (68.7% susceptible), *C. krusei* (8.6% susceptible), *C. guilliermondii* (73.5% susceptible), *C. inconspicua* (22.6% susceptible), *C. rugosa* (49.9% susceptible), *C .norvegensis* (41.9% susceptible), *C. valida* (23.8% susceptible), *C. humicola* (50% susceptible), *C. lambica* (0% susceptible), *C. ciferrii* (50% susceptible), and *C. marina* (0% susceptible) (Table 20). These data demonstrate that although fluconazole remains active against most of the common species of *Candida*, 13 of the 31 species identified in this global survey exhibit decreased susceptibility to this "workhorse" azole on the order of that seen with the well-known resistant species *C. glabrata* and *C. krusei*.

Whereas voriconazole is active against the vast majority of *Candida* spp. (Table 20), this agent is considerably less active against most fluconazole-resistant isolates (Table 21). Less than 30% of fluconazole-resistant isolates of *C. albicans* (28.1% susceptible), *C. glabrata* (19.1% susceptible), *C. tropicalis* (17.0% susceptible), *C. rugosa* (28.1% susceptible), *C. lipolytica* (29.7% susceptible), *C. pelliculosa* (16.7% susceptible), *C. lambica* (25% susceptible), and *C. apicola*, *C. haemulonis*, *C. humicola*, and *C. ciferrii* (all 0% susceptible) remained susceptible to voriconazole. Cross-resistance between fluconazole and voriconazole is clearly more pronounced in some species of *Candida* than others, although all are affected to some degree, emphasizing the importance of both species identification and antifungal susceptibility testing in the setting of candidal infection with prior azole exposure (5, 137, 180, 187, 229, 272).

Longitudinal trends over a 7-year period from 2001 to 2007 show no consistent tendency towards increasing fluconazole resistance among the common species *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, or *C. lusitaniae* on a global scale (Table 22). Likewise, consistently high levels of resistance are seen among *C. krusei*, *C. guilliermondii*, *C. rugosa*, *C. inconspicua*, and *C. norvegensis*. The global trends in fluconazole susceptibility, however, may mask the emergence of resistance that may be seen in more restricted locales, such as the different regions of the United States (Table 14).

Resistances to fluconazole and voriconazole among isolates of *C. glabrata* vary according to geographic location, ranging from lows of 13.0 and 8.2% (fluconazole and voriconazole, respectively) in the Asia-Pacific region to highs of 19.5 and 14.6% in North America (Table 23). Within each region, however, the rates of resistance varied by year from 2001 to 2007 (229). In the Asia-Pacific region, resistance to fluconazole among clinical isolates of *C. glabrata* varied from a high of 24.2% in 2001 to a low of 7.7% in 2004. Fluconazole resistance in Europe was highest in 2001 (19.3%) and 2007 (19.9%) and ranged from 14.2 to 15.7% in the intervening years. In contrast to the other regions, fluconazole resistance among Latin America isolates of *C. glabrata* increased steadily from 7.1% in 2001 to 14.9% in 2007. In North America, fluconazole resistance peaked at 25.6% in 2004 and then decreased to 13.7% in 2007.

Resistance to voriconazole increased from 6.4 to 8.6% in the Asia-Pacific region and from 3.3 to 12.0% in Latin America. There was no trend towards an increase in resistance to voriconazole among *C. glabrata* isolates from either Europe or North America (229).

The newest systemically active antifungal agents include posaconazole and the echinocandins anidulafungin, caspofungin, and micafungin. The in vitro activity of posaconazole against *Candida* spp. is comparable to that of voriconazole (Table 24). Similar to the case with voriconazole, cross resistance between posaconazole and fluconazole is well documented (223, 251). The common species *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. krusei* are very susceptible to

TABLE 20 In vitro susceptibilities of *Candida* spp. to fluconazole and voriconazole as determined by CLSI disk diffusion testing[a]

Species	Fluconazole[b]			Voriconazole[b]		
	No. of isolates tested	% S	% R	No. of isolates tested	% S	% R
C. albicans	128,625	98.0	1.4	125,965	98.5	1.2
C. glabrata	23,305	68.7	15.7	22,968	82.9	10.0
C. tropicalis	15,546	91.0	4.1	15,198	89.5	5.4
C. parapsilosis	12,788	93.2	3.6	12,453	97.0	1.8
C. krusei	5,079	8.6	78.3	5,005	83.2	7.6
C. guilliermondii	1,410	73.5	11.4	1,375	90.5	5.7
C. lusitaniae	1,233	92.1	5.4	1,215	96.7	2.0
C. kefyr	1,044	96.5	2.7	1,032	98.7	0.9
C. inconspicua	566	22.6	53.2	563	90.6	3.9
C. famata	622	79.1	10.3	606	90.3	5.0
C. rugosa	603	49.9	41.8	580	69.3	21.2
C. dubliniensis	310	96.1	2.6	308	98.4	1.0
C. norvegensis	248	41.9	40.7	247	91.5	4.0
C. lipolytica	130	66.2	28.5	128	77.3	14.1
C. sake	87	85.1	11.5	87	92.0	6.9
C. pelliculosa	87	89.7	6.9	86	94.2	4.7
C. apicola	57	98.2	1.8	57	98.20	1.8
C. zeylanoides	70	67.1	24.3	67	85.1	6.0
C. valida	21	23.8	61.9	22	81.8	13.6
C. intermedia	24	95.8	4.2	25	100.0	0.0
C. pulcherrima	14	100.0	0.0	14	100.0	0.0
C. haemulonis	9	88.9	11.1	9	88.9	11.1
C. stellatoidea	7	85.7	0.0	7	85.7	14.3
C. utilis	6	83.3	0.0	7	100.0	0.0
C. humicola	6	50.0	50.0	6	50.0	33.3
C. lambica	5	0.0	80.0	5	40.0	20
C. ciferrii	2	50.0	50.0	2	50.0	0.0
C. colliculosa	2	100.0	0.0	2	100.0	0.0
C. holmii	1	100.0	0.0	1	100.0	0.0
C. marina	1	0.0	0.0	1	100.0	0.0
C. sphaerica	1	100.0	0.0	1	100.0	0.0
Candida spp.[c]	9,744	86.2	8.9	9,577	93.6	4.1

[a]Isolates were obtained from 133 institutions from 2001 to 2007. Data compiled from reference 229.

[b]Fluconazole and voriconazole disk diffusion testing was performed in accordance with CLSI document M44-A (42). The interpretive breakpoints (zone diameters) were as follows: susceptible (S), ≥19 mm (fluconazole) and ≥17 mm (voriconazole); resistant (R), ≤14 mm (fluconazole) and ≤13 mm (voriconazole).

[c]*Candida* species, not otherwise specified.

the echinocandins, whereas these agents are considerably less potent against *C. parapsilosis*, *C. guilliermondii*, and *C. rugosa* (220, 224, 226). Although sporadic cases of acquired resistance to the echinocandins have been described (198), no consistent trend towards increased resistance to this new class of antifungals has been detected (220). The recent reports concerning increases in infections with *C. parapsilosis*, including breakthrough infections on caspofungin, are of concern and merit close observation (67, 104, 267).

Agar-based susceptibility testing methods such as Etest (AB BIODISK, Solna, Sweden) have proven to be the most sensitive and reliable methods with which to detect decreased susceptibility (resistance) to amphotericin B among *Candida* spp. (113, 150, 188, 203, 212, 292). Although interpretive breakpoints for amphotericin B have not been established, isolates of *Candida* for which MICs are >1 μg/ml are unusual and possibly "resistant" or, at the very least, may require high doses of amphotericin B for optimal treatment (187, 244, 272). Given these considerations, it is now evident that *C. glabrata*, *C. krusei*, *C. orthopsilosis*, *C. kefyr*, *C. lipolytica*, and *C. rugosa* exhibit decreased susceptibility to amphotericin B compared with *C. albicans* (Table 25) (60, 203, 212, 214). Whereas *C. guilliermondii* and *C. lusitaniae* have been described as amphotericin B-resistant *Candida* species (14, 57, 200, 215), both of these species appear to be susceptible to amphotericin B upon initial isolation from blood (Table 25). Thus, resistance to amphotericin B may develop secondarily during treatment, and repeat ampho-

TABLE 21 In vitro susceptibility of fluconazole-resistant isolates of *Candida* spp. to voriconazole as determined by CLSI disk diffusion testing[a]

Species	No. of isolates tested	% S	% SDD	% R
C. albicans	1,782	28.1	8.4	63.6
C. glabrata	3,550	19.1	21.7	59.2
C. tropicalis	629	17.0	15.3	67.7
C. parapsilosis	431	39.2	20.4	40.4
C. krusei	3,889	79.6	11.3	9.2
C. guilliermondii	157	43.9	16.6	39.5
C. lusitaniae	63	55.6	17.5	27.0
C. kefyr	27	66.7	7.4	25.9
C. inconspicua	297	83.8	10.1	6.1
C. famata	62	37.1	24.2	38.7
C. rugosa	242	28.1	21.5	50.4
C. dubliniensis	8	62.5	0.0	37.5
C. norvegensis	100	81.0	10.0	9.0
C. lipolytica	37	29.7	27.0	43.2
C. sake	9	44.4	11.1	44.4
C. pelliculosa	6	16.7	16.7	66.7
C. apicola	1	0.0	0.0	100.0
C. zeylanoides	15	46.7	26.7	26.7
C. valida	14	71.4	7.1	21.4
C. intermedia	1	100.0	0.0	0.0
C. haemulonis	1	0.0	0.0	100.0
C. humicola	3	0.0	33.3	66.7
C. lambica	4	25.0	50.0	25.0
C. ciferrii	1	0.0	100.0	0.0
Candida spp.[b]	850	47.6	14.6	37.8

[a]Isolates obtained from 133 institutions from 2001 to 2007. The zone diameters for voriconazole disk diffusion susceptibility categories were as follows: susceptible (S), ≥17 mm; susceptible dose dependent (SDD), 14 to 16 mm; resistant (R), ≤13 mm. Data compiled from reference 229.
[b]*Candida* species not otherwise identified.

tericin B susceptibility testing is recommended for patients with persistent infection with either species while on amphotericin B therapy (14, 150, 200, 215).

SUMMARY AND CONCLUSIONS

IC is an important and persistent public health problem. The incidence and mortality rates associated with this infectious disease have remained unchanged for more than two decades despite major advances in the field of antifungal therapy. Given the ever-expanding number of individuals at risk for both nosocomial and CO candidemia, it is essential that physicians keep IC high on the differential-diagnosis list when faced with a potentially infected patient. The excess LOS associated with IC carries with it significant hospital costs, to the extent that annual expenditures for IC have been estimated at more than $1 billion in the United States alone. Epidemiological studies have revealed emerging species that may vary geographically in frequency of isolation. The list of species that may cause IC is now extensive, and one can no longer ignore or dismiss previously rare, or unrecognized, species as contaminants or clinically insignificant when they are isolated from clinical material. It is also apparent that no class of antifungal agent is immune to the development of resistance. Knowledge of the local and regional epidemiology as to the prevalent species and their susceptibility to the available antifungal agents is now more important than ever. It is essential that laboratories identify clinical isolates of *Candida* to the species level and consider an orderly program of in vitro susceptibility testing to aid in therapeutic decision making. The rate of mortality attributable to IC remains high largely due to delays in the administration of appropriate antifungal therapy. Given the dearth of useful, rapid diagnostic methods, considerable effort is now being made toward developing risk stratification strategies to guide the early application of antifungal therapy with a goal to reduce candidemia-related mortality in an efficient and cost-effective manner. Although these strategies are now focused in the ICU, the emergence of CO candidemia requires that serious consideration be given to IC in patients presenting to the emergency department as well. Both clinicians and microbiologists must strive to become familiar

TABLE 22 Trends in in vitro resistance to fluconazole among *Candida* spp. determined by CLSI disk diffusion testing over a 7-year period (ARTEMIS DISK Surveillance Program, 2001 to 2007)[a]

	Isolates resistant to fluconazole (zone < 14 mm)[b]													
	2001		2002		2003		2004		2005		2006		2007	
Species	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
C. albicans	14,268	1.0	15,147	1.5	20,624	1.5	20,988	1.6	18,737	1.6	18,983	1.3	19,878	1.3
C. glabrata	2,431	18.3	2,635	14.7	3,993	16.9	3,904	14.3	3,193	15.2	3,415	14.3	3,734	16.7
C. tropicalis	1,634	3.0	1,838	6.6	2,504	5.0	2,520	3.5	2,152	3.8	2,320	3.8	2,578	3.3
C. parapsilosis	1,501	4.2	1,632	3.9	2,416	3.1	2,234	3.3	1,592	4.1	1,725	3.0	1,688	3.7
C. guilliermondii	163	11.7	239	10.5	263	8.0	237	10.1	186	14.5	156	14.7	166	13.3
C. lusitaniae	122	6.6	131	4.6	212	2.4	209	4.8	161	6.2	195	5.1	203	8.4
C. kefyr	86	2.3	87	5.7	171	2.9	183	3.8	172	2.9	153	1.3	192	1.0
C. inconspicua	30	60.0	44	47.7	113	46.9	89	58.4	78	57.7	89	53.9	123	52.0
C. norvegensis	32	43.8	18	55.6	42	26.2	43	32.6	36	38.9	48	62.5	29	27.6

[a]Isolates from all specimen types and all hospital locations in 141 institutions. Data compiled from reference 229.
[b]Fluconazole disk diffusion testing performed in accordance with CLSI guidelines M44-A (42). *n*, number of isolates.

TABLE 23 Resistance to fluconazole and voriconazole among isolates of *C. glabrata* from four geographic regions, 2001–2007[a]

Region	Antifungal agent	No. of isolates tested	% Resistant
Asia-Pacific	Fluconazole	5,629	13.0
	Voriconazole	5,515	8.2
Europe	Fluconazole	12,439	16.3
	Voriconazole	12,288	9.8
Latin America	Fluconazole	2,039	15.1
	Voriconazole	2,000	11.3
North America	Fluconazole	2,470	19.5
	Voriconazole	2,460	14.6

[a]Fluconazole and voriconazole disk diffusion testing performed in accordance with CLSI guideline M44-A (42). Data compiled from reference 229.

TABLE 24 Comparative in vitro susceptibility of clinical isolates of *Candida* spp. to posaconazole and the echinocandins determined by CLSI broth microdilution methods[a]

Species	Antifungal agent	No. tested	MIC (μg/ml)[b]			%[c]	
			Range	50%	90%	S	R/NS
C. albicans	Posaconazole	5,827	0.007–2	0.015	0.06	99.9	0.0
	Anidulafungin	2,869	0.007–2	0.03	0.06	100.0	0.0
	Caspofungin	2,869	0.007–0.5	0.03	0.06	100.0	0.0
	Micafungin	2,869	0.007–1	0.015	0.03	100.0	0.0
C. glabrata	Posaconazole	1,517	0.03–>8	1	2	79.6	8.3
	Anidulafungin	747	0.015–4	0.06	0.12	99.9	0.1
	Caspofungin	747	0.015–>8	0.03	0.06	99.9	0.1
	Micafungin	747	0.007–1	0.015	0.015	100.0	0.0
C. parapsilosis	Posaconazole	1,542	0.007–1	0.06	0.12	100.0	0.0
	Anidulafungin	759	0.03–4	2	2	92.5	7.5
	Caspofungin	759	0.015–4	0.25	1	99.9	0.1
	Micafungin	759	0.015–2	1	2	100.0	0.0
C. tropicalis	Posaconazole	1,198	0.015–2	0.06	0.12	99.9	0.0
	Anidulafungin	625	0.007–2	0.03	0.06	100.0	0.0
	Caspofungin	625	0.007–>8	0.03	0.06	99.8	0.2
	Micafungin	625	0.007–1	0.03	0.06	100.0	0.0
C. krusei	Posaconazole	305	0.03–4	0.25	1	99.0	0.3
	Anidulafungin	136	0.015–05	0.06	0.06	100.0	0.0
	Caspofungin	136	0.015–1	0.12	0.25	100	0.0
	Micafungin	136	0.015–0.25	0.06	0.12	100.0	0.0
C. lusitaniae	Posaconazole	171	0.06–1	0.06	0.12	100.0	0.0
	Anidulafungin	171	0.06–1	0.25	0.5	100.0	0.0
	Caspofungin	171	0.06–4	0.25	0.5	99.0	1.0
	Micafungin	171	0.06–1	0.12	0.25	100.0	0.0
C. guilliermondii	Posaconazole	162	0.06–2	0.25	0.5	98.0	0.0
	Anidulafungin	162	0.06–4	1	2	93.0	7.0
	Caspofungin	162	0.06–8	0.5	1	96.0	4.0
	Micafungin	162	0.06–8	0.5	1	99.0	1.0
C. rugosa	Posaconazole	16	0.06–0.25	0.06	0.25	100.0	0.0
	Anidulafungin	16	0.06–8	1	8	80.0	20.0
	Caspofungin	16	0.06–4	0.5	2	91.0	9.0
	Micafungin	16	0.06–0.25	0.06	0.25	100.0	0.0

[a]Data compiled from references 60, 213, 218, and 220.
[b]50% and 90%, MIC encompassing 50% and 90% of all isolates tested, respectively.
[c]S, susceptible at MIC breakpoints for posaconazole (≤1 μg/ml) and anidulafungin, caspofungin, and micafungin (≤2 μg/ml); R, resistant at MIC breakpoint for posaconazole (≥4 μg/ml); NS, nonsusceptible at MIC breakpoint for anidulafungin, caspofungin, and micafungin (≥4 μg/ml).

TABLE 25 Is amphotericin B uniformly active against *Candida* species?[a]

Species	No. of isolates tested	MIC (μg/ml)	
		50%	90%
C. albicans	4,195	0.5	1
C. glabrata	949	2	4
C. krusei	234	4	8
C. guilliermondii	174	0.25	0.5
C. lusitaniae	171	0.25	0.5
C. orthopsilosis	102	1	2
C. dubliniensis	101	0.25	0.5
C. kefyr	74	1	2
C. pelliculosa	40	0.5	1
C. famata	16	0.5	0.5
C. metapsilosis	30	0.5	1
C. lipolytica	16	2	4
C. rugosa	16	2	2

[a]Data compiled from references 60, 202, 209, and 212.

with the various species of *Candida*, their epidemiological and pathogenic features, and the optimal approaches to diagnosis and therapy in order to make inroads in the management of these difficult infections. Fertile areas of research include rapid diagnostic measures, risk stratification, and assessment of management strategies (i.e., optimal timing, dose, and duration of antifungal therapy) for IC.

REFERENCES

1. **Abi-Said, D., E. Anaissie, O. Uzun, I. Raad, H. Pinzcowski, and S. Vartivarian.** 1997. The epidemiology of hematogenous candidiasis caused by different *Candida* species. *Clin. Infect. Dis.* **24:**1122–1128.
2. **Agvald-Ohman, C., L. Klingspor, H. Hjelmquist, and C. Edlund.** 2007. Invasive candidiasis in long-term patients at a multidisciplinary intensive care unit: *Candida* colonization index, risk factors, treatment and outcome. *Scand. J. Infect. Dis.* **40:**145–153.
3. **Ahlquist, A., M. M. Farley, L. H. Harrison, W. Baughman, B. Siegel, R. Hollick, S. R. Lockhart, S. S. Magill, and T. Chiller.** 2009. Epidemiology of candidemia in metropolitan Atlanta and Baltimore City and County: preliminary results of population-based active, laboratory surveillance—2008-2009, abstr. M-1241. *49th Intersci. Conf. Antimicrob. Agents Chemother.*
4. **Alcoba-Florez, J., M. del Pilar Arevalo, F. J. Gonzalez-Paredes, J. Cano, J. Guano, E. Perez-Roth, and S. Mendez-Alvarez.** 2005. PCR protocol for specific identification of *Candida nivariensis*, a recently described pathogenic yeast. *J. Clin. Microbiol.* **43:**6194–6196.
5. **Alexander, B. D., W. A. Schell, J. L. Miller, G. D. Long, and J. R. Perfect.** 2005. *Candida glabrata* fungemia in transplant patients receiving voriconazole after fluconazole. *Transplantation* **80:**868–871.
6. **Alexander, B. D., and M. A. Pfaller.** 2006. Contemporary tools for the diagnosis and management of invasive mycoses. *Clin. Infect. Dis.* **43:**515–527.
7. **Almirante, B., D. Rodriguez, B. J. Park, M. Cuenca-Estrella, A. M. Planes, M. Almela, J. Mensa, F. Sanchez, J. Ayats, M. Gimenez, P. Saballs, S. K. Fridkin, J. Morgan, J. L. Rodriguez-Tudela, D. W. Warnock, A. Pahissa, and the Barcelona Candidemia Project Study Group.** 2005. Epidemiology and predictors of mortality in cases of *Candida* bloodstream infection: results from population-based surveillance, Barcelona, Spain, from 2002 to 2003. *J. Clin. Microbiol.* **43:**1829–1835.
8. **Antoniadou, A., H. A. Torres, R. E. Lewis, J. Thornby, G. P. Bodey, J. P. Tarrand, X. Y. Han, K. V. Rolston, A. Safdar, I. I. Raad, and D. P. Kontoyiannis.** 2003. Candidemia in a tertiary care center: in vitro susceptibility and its association with outcome of initial antifungal therapy. *Medicine* **82:**309–321.
9. **Arendrup, M. C., K. Fuursted, B. Gahrn-Hansen, I. M. Jensen, J. D. Knudsen, B. Lundgren, H. C. Schonheyder, and M. Tvede.** 2005. Seminational surveillance of fungemia in Denmark: notably high rates of fungemia and numbers of isolates with reduced azole susceptibility. *J. Clin. Microbiol.* **43:**4434–4440.
10. **Arendrup, M. C., K. Fuursted, B. Gahrn-Hansen, H. C. Schonheyder, J. D. Knudsen, I. M. Jensen, B. Bruun, J. J. Christensen, and H. K. Johansen.** 2008. Semi-national surveillance of candidemia in Denmark 2004–2006: increasing incidence of fungemia and numbers of isolates with reduced azole susceptibility. *Clin. Microbiol. Infect.* **14:**487–494.
11. **Armstrong-James, D.** 2007. Invasive *Candida* species infection: the importance of adequate empirical antifungal therapy. *J. Antimicrob. Chemother.* **60:**459–460.
12. **Asmundsdottir, L. R., H. Erlendsdottir, and M. Gottfredsson.** 2002. Increasing incidence of candidemia: results from a 20-year nationwide study in Iceland. *J. Clin. Microbiol.* **40:**3489–3492.
13. **Asmundsdottir, L. R., H. Erlendsdottir, G. Haraldsson, H. Guo, J. Xu, and M. Gottfredsson.** 2008. Molecular epidemiology of candidemia: evidence of clusters of smoldering nosocomial infections. *Clin. Infect. Dis.* **47:**e17–e24.
14. **Atkinson, B. J., R. E. Lewis, and D. P. Kontoyiannis.** 2008. *Candida lusitaniae* fungemia in cancer patients: risk factors for amphotericin B failure and outcome. *Med. Mycol.* **46:**541–546.
15. **Baddley, J. W., A. M. Smith, S. A. Moser, and P. E. Pappas.** 2001. Trends in frequency and susceptibilities of *Candida glabrata* bloodstream isolates at a university hospital. *Diagn. Microbiol. Infect. Dis.* **39:**199–201.
16. **Bai, F. Y.** 1996. Separation of *Candida fermentati* comb. nov. from *Candida guilliermondii* by DNA base composition and electrophoretic karyotyping. *Syst. Appl. Microbiol.* **19:**178–181.
17. **Baily, G. G., C. B. Moore, S. M. Essayag, S. de Wit, J. P. Burnie, and D. W. Denning.** 1997. *Candida inconspicua*, a fluconazole-resistant pathogen in patients infected with human immunodeficiency virus. *Clin. Infect. Dis.* **25:**161–163.
18. **Baran, J., Jr., B. Buckatira, and R. Khatib.** 2001. Candidemia before and during the fluconazole era: prevalence, types of species, and approach to treatment in a tertiary care community hospital. *Scand. J. Infect. Dis.* **33:** 137–139.
19. **Barchiesi, F., E. Spreghini, S. Tomassettii, A. D. Vittoria, D. Arzeni, E. Manso, and G. Scalise.** 2006. Effects of caspofungin against *Candida guilliermondii* and *Candida parapsilosis*. *Antimicrob. Agents Chemother.* **50:**2719–2727.
20. **Bennett, J. E., K. Izumikawa, and K. A. Marr.** 2004. Mechanism of increased fluconazole resistance in *Candida glabrata* during prophylaxis. *Antimicrob. Agents Chemother.* **48:**1773–1777.
21. **Bilgen, H., E. Ozek, V. Korten, B. Ener, D. Molbay.** 1995. Treatment of systemic neonatal candidiasis with fluconazole. *Infection* **23:**394.

22. **Bishop, J. A., N. Chase, S. S. Magill, C. P. Kurtzman, M. J. Fiandara, and W. G. Merz.** 2008. *Candida bracarensis* detected among isolates of *Candida glabrata* by peptide nucleic acid fluorescence in situ hybridization: susceptibility data and documentation of presumed infection. *J. Clin. Microbiol.* **46:**443–446.
23. **Blinkhorn, R. J., D. Adelstein, and P. J. Spagnuolo.** 1989. Emergence of a new opportunistic pathogen, *Candida lusitaniae*. *J. Clin. Microbiol.* **27:**236–240.
24. **Bliss, J. M., K. P. Basavegowda, W. J. Watson, A. U. Sheikh, and R. M. Ryan.** 2008. Vertical and horizontal transmission of *Candida albicans* in very low birth weight infants using DNA fingerprinting techniques. *Pediatr. Infect. Dis. J.* **27:**231–235.
25. **Blot, S. I., K. H. Vandewoude, E. A. Hoste, and F. A. Colandyn.** 2002. Effects of nosocomial candidemia on outcomes of critically ill patients. *Am. J. Med.* **113:**480–485.
26. **Blumberg, H. M., W. R. Jarvis, J. M. Soucie, J. E. Edwards, J. E. Patterson, M. A. Pfaller, M. S. Rangel-Frausto, M. G. Rinaldi, L. Saiman, R. T. Wiblin, R. P. Wenzel, and the NEMIS Study Group.** 2001. Risk factors for candidal bloodstream infections in surgical intensive care unit patients: the NEMIS prospective multicenter study. *Clin. Infect. Dis.* **33:**177–186.
27. **Blyth, C. C., S. C. A. Chen, M. A. Slavin, C. Serena, Q. Nguyen, D. Marriott, D. Ellis, W. Meyer, T. C. Sorrell, and the Australian Candidemia Study.** 2009. Not just little adults: candidemia epidemiology, molecular characterization, and antifungal susceptibility in neonatal and pediatric patients. *Pediatrics* **123:**1360–1368.
28. **Bochud, P. Y., M. Bonter, O. Marchetti, and T. Calandra.** 2004. Antimicrobial therapy for patients with severe sepsis and septic shock: an evidence-based review. *Crit. Care Med.* **32**(Suppl.)**:**S495–S512.
29. **Bonassoli, L. A., M. Bertoli, and T. I. Suidsinzki.** 2005. High frequency of *Candida parapsilosis* on the hands of healthy hosts. *J. Hosp. Infect.* **59:**159–162.
30. **Borg-von Zeplin, M., L. Kunz, R. Ruchel, U. Reichard, M. Weig, and U. Grop.** 2007. Epidemiology and antifungal susceptibilities of *Candida* spp. to six antifungal agents: results from a surveillance study on fungemia in Germany from July 2004 to August 2005. *J. Antimicrob. Chemother.* **60:**424–428.
31. **Borman, A. M., R. Petch, C. J. Linton, M. D. Palmer, P. D. Bridge, and E. M. Johnson.** 2008. *Candida nivariensis*, an emerging pathogenic fungus with multidrug resistance to antifungal agents. *J. Clin. Microbiol.* **46:**933–938.
32. **Borst, A., M. T. Raimer, D. W. Warnock, C. J. Morrison, and B. A. Arthington-Skaggs.** 2005. Rapid acquisition of stable azole resistance by *Candida glabrata* isolates obtained before the clinical introduction of fluconazole. *Antimicrob. Agents Chemother.* **49:**783–787.
33. **Brun, S., T. Berges, P. Poupard, C. Vauzelle-Moreau, G. Renier, D. Chabasse, and J. P. Bouchara.** 2004. Mechanism of azole resistance in petite mutants of *Candida glabrata*. *Antimicrob. Agents Chemother.* **48:**1788–1796.
34. **Chakrabarti, A., S. Chatterjee, K. L. N. Rao, M. M. Zameer, M. R. Shivaprakash, S. Singh, R. Singh, and S. C. Varma.** 2009. Recent experience with fungemia: change in species distribution and azole resistance. *Scand. J. Infect. Dis.* **41:**275–284.
35. **Charles, P. E.** 2006. Multifocal *Candida* species colonization as a trigger for early antifungal therapy in critically ill patients: what about other risk factors for fungal infection? *Crit. Care Med.* **34:**913–914.
36. **Chen, S. C. A., D. Marriott, E. G. Playford, Q. Nguyen, D. Ellis, W. Meyer, T. C. Sorrell, M. Slavin, and the Australian Candidemia Study.** 2009. Candidemia with uncommon *Candida* species: predisposing factors, outcome, antifungal susceptibility, and implications for management. *Clin. Microbiol. Infect.* **15:**662–669.
37. **Chen, T. C., Y. H. Chen, J. J. Tsai, C. F. Peng, P. L. Lu, K. Chang, H. C. Hsieh, and T. P. Chen.** 2005. Epidemiologic analysis and antifungal susceptibility of *Candida* blood isolates in southern Taiwan. *J. Microbiol. Immunol. Infect.* **38:**200–210.
38. **Chen, Y.-C., S.-C. Chang, K.-T. Luh, and W.-C. Hsieh.** 2003. Stable susceptibility of *Candida* blood isolates to fluconazole despite increasing use during the past 10 years. *J. Antimicrob. Chemother.* **52:**71–77.
39. **Cheng, M. F., K. W. Yu, R. B. Tang, Y. H. Fan, Y. L. Yang, K. S. Hsieh, M. Ho, and H. J. Lo.** 2004. Distribution and antifungal susceptibility of *Candida* species causing candidemia from 1996 to 1999. *Diagn. Microbiol. Infect. Dis.* **48:**33–37.
40. **Chow, J. K., Y. Golan, R. Ruthazer, A. W. Karchmer, Y. Carmeli, D. Lichtenberg, V. Chawea, J. Young, and S. Hadley.** 2008. Factors associated with candidemia caused by non-*albicans Candida* species versus *Candida albicans* in the intensive care unit. *Clin. Infect. Dis.* **46:**1206–1213.
41. **Clark, T. A., S. A. Slavinski, J. Morgan, T. Lott, B. A. Artington-Skaggs, M. E. Brandt, R. M. Webb, M. Carrier, R. H. Flowers, S. K. Fridkin, and R. A. Hajjeh.** 2004. Epidemiologic and molecular characterization of an outbreak of *Candida parapsilosis* bloodstream infections in a community hospital. *J. Clin. Microbiol.* **42:**4468–4472.
42. **Clinical and Laboratory Standards Institute.** 2004. *Method for Antifungal Disk Diffusion Testing of Yeasts: Approved Standard, M44-A*. Clinical and Laboratory Standards Institute, Wayne, PA.
43. **Cohen, J., C. Brun-Buisson, A. Torres, and J. Jorgensen.** 2004. Diagnosis of infection in sepsis: an evidence-based review. *Crit. Care Med.* **32**(Suppl.)**:**S466–S494.
44. **Coleman, D. C., D. J. Sullivan, D. E. Bennett, G. P. Morgen, H. J. Barry, and D. B. Shanley.** 1997. Candidiasis: the emergence of a novel species, *Candida dubliniensis*. *AIDS* **11:**557–567.
45. **Collin, B., C. J. Clancy, and M. H. Nguyen.** 1999. Antifungal resistance in non-*albicans Candida* species. *Drug Resist. Updat.* **2:**9–14.
46. **Colombo, A. L., M. Nucci, R. Salomao, M. L. M. Branchini, R. Richtmann, A. Derossi, and S. B. Wey.** 1999. High rate of non-*albicans* candidemia in Brazilian tertiary care hospitals. *Diagn. Microbiol. Infect. Dis.* **34:**281–286.
47. **Colombo, A. L., A. S. A. Melo, R. F. C. Rosas, R. Salomao, M. Briones, R. J. Hollis, S. A. Messer, and M. A. Pfaller.** 2003. Outbreak of *Candida rugosa* candidemia: an emerging pathogen that may be refractory to amphotericin B therapy. *Diagn. Microbiol. Infect Dis.* **46:**253–257.
48. **Colombo, A. L., J. Perfect, M. DiNubile, K. Bartizal, M. Motyl, P. Hicks, R. Lupinacci, C. Sable, and N. Kartsonis.** 2003. Global distribution and outcomes for *Candida* species causing invasive candidemia: results from an international randomized double-blind study of caspofungin versus amphotericin B for the treatment of invasive candidiasis. *Eur. J. Clin. Microbiol. Infect. Dis.* **22:**470–474.
49. **Colombo, A. L., M. Nucci, B. J. Park, S. A. Nouer, B. Arthington-Skaggs, D. A. da Matta, D. Warnock, and J. Morgan for the Brazilian Network Candidemia Study.** 2006. Epidemiology of candidemia in Brazil: a nationwide sentinel surveillance of candidemia in eleven medical centers. *J. Clin. Microbiol.* **44:**2816–2823.
50. **Corona, A., A. O. L. Sacco, and M. Singer.** 2009. Early-onset candidemia: an increasing problem? *Crit. Care Med.* **37:**2652–2653.

51. **Correia, A., P. Sampaio, S. James, and C. Pais.** 2006. *Candida bracarensis* sp. nov., a novel anamorphic yeast species phenotypically similar to *Candida glabrata*. *Int. J. Syst. Evol. Microbiol.* **56:**313–317.
52. **Cuenca-Estrella, M., A. Gomez-Lopez, E. Mellado, M. J. Buitrago, A. Monzon, and J. L. Rodriguez-Tudela.** 2006. Head-to-head comparison of the activities of currently available antifungal agents against 3,378 Spanish clinical isolates of yeast and filamentous fungi. *Antimicrob. Agents Chemother.* **50:**917–921.
53. **da Matta, D. A., L. P. de Almeida, A. M. Machado, A. C. Azevedo, E. J. U. Kusano, N. F. Travassos, R. Salomao, and A. L. Colombo.** 2007. Antifungal susceptibility of 1000 *Candida* bloodstream isolates to 5 antifungal drugs: results of a multicenter study conducted in São Paulo, Brazil, 1995–2003. *Diagn. Microbiol. Infect. Dis.* **57:**399–404.
54. **D'Antonio, D., B. Violante, A. Mazzoni, T. Bonfini, M. A. Capuani, F. D'Aloia, A. Iacune, F. Schioppa, and F. Romano.** 1998. A nosocomial cluster of *Candida inconspicua* infections in patients with hematological malignancies. *J. Clin. Microbiol.* **36:**792–795.
55. **Davies, H. D., A. McGeer, B. Schwartz, K. Green, D. Can, A. E. Simon, and D. E. Low for the Ontario Group A Streptococcal Study Group.** 1996. Invasive group A streptococcal infections in Ontario, Canada. *N. Engl. J. Med.* **335:**547–554.
56. **Dellinger, R. P., M. M. Levy, J. M. Carlet, J. Bion, M. M. Parker, R. Jaeschke, K. Reinhart, D. C. Angus, C. Brun-Buisson, R. Beale, T. Calandra, J. F. Dhainaut, H. Gerlach, M. Harvey, J. J. Marini, J. Marshall, M. Ranieri, G. Ramsay, J. Sevransky, B. T. Thompson, S. Townsend, J. S. Vender, J. L. Zimmerman, and J. L. Vincent.** 2008. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. *Intensive Care Med.* **34:**17–60.
57. **Dick, J. D., R. P. Rosengard, W. G. Merz, R. K. Stuart, G. M. Hutchins, and R. Saral.** 1985. Fatal disseminated candidiasis due to amphotericin B-resistant *Candida guilliermondii*. *Ann. Intern. Med.* **102:**67–68.
58. **Diekema, D. J., S. A. Messer, A. B. Brueggemann, S. H. Coffman, G. V. Doern, L. A. Herwaldt, and M. A. Pfaller.** 2002. Epidemiology of candidemia: 3-year results from the Emerging Infections and the Epidemiology of Iowa Organisms Study. *J. Clin. Microbiol.* **40:**1298–1302.
59. **Diekema, D. J., and M. A. Pfaller.** 2004. Nosocomial candidemia: an ounce of prevention is better than a pound of cure. *Infect. Control Hosp. Epidemiol.* **25:**624–626.
60. **Diekema, D. J., S. A. Messer, L. B. Boyken, R. J. Hollis, J. Kroeger, S. Tendolkar, and M. A. Pfaller.** 2009. In vitro activity of seven systemically active antifungal agents against a large global collection of rare *Candida* species as determined by CLSI broth microdilution methods. *J. Clin. Microbiol.* **47:**3170–3177.

60a. **Diekema, D. J., S. A. Messer, R.J. Hollis, L. Boyken, S. Tendolkar, J. Kroeger, R. N. Jones, and M. A. Pfaller.** 2009. A global evaluation of voriconazole activity tested against recent clinical isolates of *Candida* spp. *Diagn. Microbiol. Infect. Dis.* **63:**233–236.

61. **Dimopoulos, G., A. Velegraki, and M. E. Falagas.** 2009. Ten year survey on antifungal susceptibility of candidemia isolates from intensive care unit patients in Greece. *Antimicrob. Agents Chemother.* **53:**1242–1244.
62. **Dube, M. P., P. N. Heseltine, M. G. Rinaldi, S. Evans, and B. Zawacki.** 1994. Fungemia and colonization with nystatin-resistant *Candida rugosa* in a burn unit. *Clin. Infect. Dis.* **18:**77–82.
63. **Eggimann, P., J. Garbino, and D. Pittet.** 2003. Epidemiology of *Candida* species infections in critically ill non-immunosuppressed patients. *Lancet Infect. Dis.* **3:** 685–702.
64. **Enache-Angoulvant, A., A. Girard, J. L. Poriot, and C. Hennequin.** 2009. In vitro activity of caspofungin and voriconazole against uncommon *Candida* spp. *Int. J. Antimicrob. Agents* **33:**595–596.
65. **Falagas, M. E., K. E. Apostolou, and V. D. Pappas.** 2006. Attributable mortality of candidemia: a systematic review of matched cohort and case-control studies. *Eur. J. Clin. Microbiol. Infect Dis.* **25:**419–425.
66. **Farley, M. M., R. C. Harvey, T. Stull, J. D. Smith, A. Shuchat, J. D. Wenger, and D. S. Stephens.** 1993. A population-based assessment of invasive disease due to group B *Streptococcus* in nonpregnant adults. *N. Engl. J. Med.* **328:**1807–1811.
67. **Forrest, G. N., E. Weekes, and J. K. Johnson.** 2008. Increasing incidence of *Candida parapsilosis* candidemia with caspofungin use. *J. Infect.* **56:**126–129.
68. **Fridkin, S. K.** 2005. Candidemia is costly—plain and simple. *Clin. Infect. Dis.* **41:**1240–1241.
69. **Fridkin, S. K.** 2005. The changing face of fungal infections in health care settings. *Clin. Infect. Dis.* **41:**1455–1460.
70. **Fridkin, S. K., D. Kaufman, J. R. Edwards, S. Shetty, T. Horan, and the National Nosocomial Infections Surveillance System Hospitals.** 2006. Changing incidence of *Candida* bloodstream infections among NICU patients in the United States: 1995–2004. *Pediatrics* **117:**1680–1687.
71. **Fujita, S. I., Y. Senda, T. Okusi, Y. Ota, H. Takada, K. Yamada, and M. Kawano.** 2007. Catheter-related fungemia due to fluconazole-resistant *Candida nivariensis*. *J. Clin. Microbiol.* **45:**3459–3461.
72. **Fukuoka, T., D. A. Johnston, C. A. Winslow, M. J. de Groot, C. Burt, C. A. Hitchock, and S. G. Filler.** 2003. Genetic basis for differential activities of fluconazole and voriconazole against *Candida krusei*. *Antimicrob. Agents Chemother.* **47:**1213–1219.
73. **Gales, A. C., M. A. Pfaller, A. K. Houston, S. Joly, D. J. Sullivan, D. C. Coleman, and D. R. Soll.** 1999. Identification of *Candida dubliniensis* based on temperature and utilization of xylose and α-methyl-D-glucose as determined with the API 20C AUX and Vitek YBC systems. *J. Clin. Microbiol.* **37:**3804–3808.
74. **Garey, K. W., M. Rege, M. P. Pai, D. E. Mingo, K. J. Suda, R. S. Turpin, and D. T. Bearden.** 2006. Time to initiation of fluconazole therapy impacts mortality in patients with candidemia: a multi-institutional study. *Clin. Infect. Dis.* **43:**25–31.
75. **Garey, K. W., M. P. Pai, K. J. Suda, R. S. Turpin, M. D. Rege, D. E. Mingo, and D. T. Bearden.** 2007. Inadequacy of fluconazole dosing in patients with candidemia based on Infectious Diseases Society of America (IDSA) guidelines. *Pharmacoepidemiol. Drug Saf.* **16:**919–927.
76. **Garrouste-Orgeas, M., J. F. Timsit, M. Tafflet, B. Misset, J. R. Zahar, L. Soufir, T. Lazard, S. Jamali, B. Mourvillier, Y. Cohen, A. De Lassence, E. Azoulay, C. Cheval, A. Descrops-Declere, C. Adrie, M. A. Costa de Beauregard, and J. Claret for the OUTCOMEREA Study Group.** 2006. Excess risk of death from intensive care unit-acquired nosocomial bloodstream infections: a reappraisal. *Clin. Infect. Dis.* **42:**1118–1126.
77. **Girmenia, C., G. Pizzarelli, F. Cristini, F. Barchiesi, E. Spreghini, G. Scalise, and P. Martino.** 2006. *Candida guilliermondii* fungemia in patients with hematologic malignancies. *J. Clin. Microbiol.* **44:**2458–2464.
78. **Godoy, P., I. N. Tiraboschi, L. C. Servero, B. Bustamonte, B. Calvo, L. P. de Almedia, D. A. da Matta, and A. L. Colombo.** 2003. Species distribution and antifungal

susceptibility profile of *Candida* spp. bloodstream isolates from Latin American hospitals. *Mem. Inst. Oswaldo Cruz* **98:**401–405.
79. **Goldman, M., G. A. Cloud, M. Smedema, A. Le Monte, P. Connolly, D. S. McKinsey, C. A. Kauffman, B. Moskovitz, and L. J. Wheat.** 2000. Does long-term itraconazole prophylaxis result in in vitro azole resistance in mucosal *Candida albicans* isolates from persons with advanced human immunodeficiency virus infection? *Antimicrob. Agents Chemother.* **44:**1585–1587.
80. **Gonzalez, G. M., M. Elizondo, and J. Ayala.** 2008. Trends in species distribution and susceptibility of bloodstream isolates of *Candida* collected in Monterrey, Mexico, to seven antifungal agents: results of a 3-year (2004 to 2007) surveillance study. *J. Clin. Microbiol.* **46:**2902–2905.
81. **Gudlaugsson, O., S. Gillespie, K. Lee, J. Vande Berg, J. Hu, S. Messer, L. Herwaldt, M. Pfaller, and D. Diekema.** 2003. Attributable mortality of nosocomial candidemia, revisited. *Clin. Infect. Dis.* **37:**1172–1177.
82. **Hachem, R., H. Hanna, D. Kontoyiannis, Y. Jiang, and I. Raad.** 2008. The changing epidemiology of invasive candidiasis: *Candida glabrata* and *Candida krusei* as the leading causes of candidemia in hematologic malignancy. *Cancer* **112:**2493–2499.
83. **Hajjeh, R. A., A. N. Sofair, L. H. Harrison, G. M. Lyon, B. A. Arthington-Skaggs, S. A. Mirza, M. Phelan, J. Morgan, W. Lee-Yong, M. A. Ciblak, L. E. Benjamin, L. T. Sanza, S. Huie, S. F. Yeo, M. E. Brandt, and D. W. Warnock.** 2004. Incidence of bloodstream infections due to *Candida* species and in vitro susceptibilities of isolates collected from 1998 to 2000 in a population-based active surveillance program. *J. Clin. Microbiol.* **42:**1519–1527.
84. **Harbarth, S., K. Kerriere, S. Hugonetti, B. Ricou, P. Sutter, and D. Pittet.** 2002. Epidemiology and prognostic determinants of bloodstream infections in surgical intensive care. *Arch. Surg.* **137:**1353–1359.
85. **Hassan, I., G. Powell, M. Sidhu, W. M. Hart, and D. W. Denning.** 2009. Excess mortality, length of stay and cost attributable to candidemia. *J. Infect.* **59:**360–365.
86. **Hawkins, J. L., and L. M. Baddour.** 2003. *Candida lusitaniae* infections in the era of fluconazole availability. *Clin. Infect. Dis.* **36:**e14–e18.
87. **Hazen, K. C.** 1995. New and emerging yeast pathogens. *Clin. Microbiol. Rev.* **8:**462–478.
88. **Hazen, K. C., and S. A. Howell.** 2007. *Candida*, *Cryptococcus*, and other yeasts of medical importance, p. 1762–1788. *In* P. R. Murray, E. J. Baron, J. H. Jorgensen, M. L. Landry, and M. A. Pfaller (ed.), *Manual of Clinical Microbiology*, 9th ed. ASM Press, Washington, DC.
89. **Hedderwick, S. A., J. Y. Wan, S. F. Bradley, J. A. Sangeorzan, M. S. Perpening, and C. A. Kauffman.** 1998. Risk factors for colonization with yeast species in a Veterans Affairs long-term care facility. *J. Am. Geriatr. Soc.* **46:**849–853.
90. **Holzshu, D. L., H. L. Presley, M. Miranda, and H. J. Phaff.** 1979. Identification of *Candida lusitaniae* as opportunistic yeasts in humans. *J. Clin. Microbiol.* **10:**202–205.
91. **Hood, S. V., C. B. Moore, and D. W. Denning.** 1996. Isolation of *Candida norvegensis* from clinical specimens: four case reports. *Clin. Infect. Dis.* **23:**1185–1187.
92. **Hope, W., A. Morton, and D. P. Eisen.** 2002. Increase in prevalence of nosocomial non-*Candida albicans* candidemia and the association of *Candida krusei* with fluconazole use. *J. Hosp. Infect.* **50:**56–65.
93. **Horn, D., D. Neofytos, J. Fishman, W. Steinbach, E. Anaisie, K. A. Marr, M. Pfaller, A. Olyaei.** 2007. Use of the PATH Alliance database to measure adherence to IDSA guidelines for the therapy of candidemia. *Eur. J. Clin. Microbiol. Infect. Dis.* **26:**907–914.
94. **Horn, D. L., D. Neofytos, E. J. Anaissie, J. A. Fishman, W. J. Steinbach, A. J. Olyaei, K. A. Marr, M. A. Pfaller, C. H. Chang, and K. M. Webster.** 2009. Epidemiology and outcomes of candidemia in 2019 patients: data from the Prospective Antifungal Therapy Alliance Registry. *Clin. Infect. Dis.* **48:**1695–1703.
95. **Horvath, L. L., B. J. George, C. K. Murray, L. S. Harrison, and D. R. Hospenthal.** 2004. Direct comparison of the BACTEC 9320 and BacT/ALERT 3D automated blood culture systems for *Candida* growth detection. *J. Clin. Microbiol.* **42:**115–118.
96. **HPA.** 2009. *Candidemia in England, Wales, and Northern Ireland: 2007—Overall Trend in Reports*. HPA, London, England. http://www.hpa.org.uk. (Accessed 28 January 2010.)
97. **Hsueh, P. R., L. J. Teng, P. C. Yang, S. W. Ho, and K. T. Luh.** 2002. Emergence of nosocomial candidemia at a teaching hospital in Taiwan from 1981 to 2000: increased susceptibility of *Candida* species to fluconazole. *Microb. Drug Resist.* **8:**311–319.
98. **Hsueh, P. R., Y. J. Lau, Y. C. Chuang. J. H. Wan, W. K. Huang, J. M. Syr, J. J. Yan, K. W. Yu, J. J. Wu, W. C. Ko, Y. C. Yang, Y. C. Liu, L. J. Teng, C. Y. Lin, and K. T. Luh.** 2005. Antifungal susceptibilities of clinical isolates of *Candida* species, *Cryptococcus neoformans*, and *Aspergillus* species from Taiwan: surveillance of multicenter antimicrobial resistance in Taiwan program data from 2003. *Antimicrob. Agents Chemother.* **49:**512–517.
99. **Ibrahim, E. H., G. Sherman, S. Ward, V. J. Fraser, and M. H. Kollef.** 2000. The influence of inadequate antimicrobial treatment of bloodstream infections on patient outcomes in the ICU setting. *Chest* **118:**146–155.
100. **Iwen, P. C., D. M. Kelly, E. C. Reed, and S. H. Hinrichs.** 1995. Invasive infections due to *Candida krusei* in immunocompromised patients not treated with fluconazole. *Clin. Infect. Dis.* **20:**342–347.
101. **Jarvis, W. R.** 1995. Epidemiology of nosocomial fungal infections with emphasis on *Candida* species. *Clin. Infect. Dis.* **20:**1526–1530.
102. **Jarvis, W. R., and W. J. Martone.** 1992. Predominant pathogens in hospital infections. *J. Antimicrob. Chemother.* **29**(Suppl. A)**:**19–24.
103. **Johnson, E. M.** 2009. Rare and emerging *Candida* species. *Curr. Fungal Infect. Rep.* **3:**152–159.
104. **Kabbara, N., C. Lacroix, R. Peffault de Latour, G. Socie, M. Ghannoum, and P. Ribaud.** 2008. Breakthrough *C. parapsilosis* and *C. guilliermondii* bloodstream infections in allogeneic hematopoietic stem cell transplant recipients receiving long-term caspofungin therapy. *Haematologica* **93:**639–640.
105. **Kao, A. S., M. E. Brandt, W. R. Pruit, L. A. Conn, B. A. Perkins, D. S. Stephens, W. S. Baughman, A. L. Reingold, G. A. Rothrock, M. A. Pfaller, R. W. Pinner, and R. A. Hajjeh.** 1999. The epidemiology of candidemia in two United States cities: results of a population-based active surveillance. *Clin. Infect. Dis.* **29:**1164–1170.
106. **Kauffman, C. A.** 2001. Fungal infections in older adults. *Clin. Infect. Dis.* **33:**550–555.
107. **Kaufman, D., R. Boyle, K. C. Hazen, J. T. Patrie, M. Robinson, and L. G. Donowitz.** 2001. Fluconazole prophylaxis against fungal colonization and infection in preterm infants. *N. Engl. J. Med.* **345:**1660–1666.
108. **Klevay, M. J., E. J. Ernst, J. L. Hollenbaugh, J. G. Miller, M. A. Pfaller, and D. J. Diekema.** 2008. Therapy and outcome of *Candida glabrata* versus *Candida albicans* bloodstream infection. *Diagn. Microbiol. Infect. Dis.* **60:**273–277.

109. **Klevay, M. J., D. L. Horn, D. Neofytos, M. A. Pfaller, and D. J. Diekema.** 2009. Initial treatment and outcome of *Candida glabrata* versus *C. albicans* bloodstream infection. *Diagn. Microbiol. Infect. Dis.* **64:**152–157.
110. **Kollef, M. H., L. M. Napolitino, J. S. Solomkin, R. G. Wunderink, I. G. Bae, V. G. Fowler, R. A. Balk, D. L. Stevens, J. J. Rahal, A. F. Shorr, P. K. Linden, and S. T. Micek.** 2008. Health care-associated infection (HAI): a critical appraisal of the emerging threat—proceedings of the HAI Summit. *Clin. Infect. Dis.* **47**(Suppl. 2)**:** S55–S99.
111. **Kontoyiannis, D. P., I. Vaziri, H. A. Hanna, M. Boktour, J. Thornby, R. Hachem, G. P. Bodey, and I. I. Raad.** 2001. Risk factors for *Candida tropicalis* fungemia in patients with cancer. *Clin. Infect. Dis.* **33:**1676–1681.
112. **Krcmery, V., S. Grausova, M. Mraz, E. Pichova, and L. Jurga.** 1999. *Candida guilliermondii* fungemia in cancer patients: report of three cases. *J. Infect.* **5:**58–59.
113. **Krogh-Madsen, M., M. C. Arendrup, L. Heslet, and J. D. Knudsen.** 2006. Amphotericin B and caspofungin resistance in *Candida glabrata* isolates recovered from a critically ill patient. *Clin. Infect. Dis.* **42:**938–944.
114. **Kuhn, D. M., P. K. Mukherjee, T. A. Clark, C. Pujol, J. Chandra, R. A. Hajjeh, D. W. Warnock, D. R. Soll, and M. A. Ghannoum.** 2004. *Candida parapsilosis* characterization in an outbreak setting. *Emerg. Infect. Dis.* **10:**1074–1081.
115. **Kumar, A., D. Roberts, K. E. Woods, B. Light, J. E. Parrillo, S. Sharma, R. Suppes, D. Feinstein, S. Zanotti, L. Taiberg, D. Gurka, A. Kumar, and M. Cheang.** 2006. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Crit. Care Med.* **34:** 1589–1596.
116. **Lan, L., and J. Xu.** 2006. Multiple gene genealogical analyses suggest divergence and recent clonal dispersal in the opportunistic human pathogen *Candida guilliermondii*. *Microbiology* **152:**1539–1549.
117. **Lattif, A. A., P. Mukherjee, J. Chandra, K. Swindell, S. R. Lockhart, D. J. Diekema, M. A. Pfaller, and M. A. Ghannoum.** 2010. Characterization of biofilms formed by *Candida parapsilosis*, *C. metapsilosis*, and *C. orthopsilosis*. *Int. J. Med. Microbiol.* **300:**265–270.
118. **Laupland, K. B., D. B. Gregson, D. L. Church, T. Ross, and S. Elsayed.** 2005. Invasive *Candida* species infection: a 5 year population-based assessment. *J. Antimicrob. Chemother.* **56:**532–537.
119. **Laverdiere, M., C. Rotstein, E. J. Bow, R. S. Robert, S. Ioannou, D. Carr, and N. Moghaddam.** 2000. Impact of fluconazole prophylaxis on fungal colonization and infection rates in neutropenic patients. *J. Antimicrob. Chemother.* **46:**1001–1008.
120. **Lee, I., N. O. Fishman, T. E. Zaoutis, K. H. Morales, M. G. Weiner, M. Synnestvedt, I. Nachamkin, and E. Lautenbach.** 2009. Risk factors for fluconazole-resistant *Candida glabrata* bloodstream infections. *Arch. Intern. Med.* **169:**379–383.
121. **Lee, J. S., J. H. Shin, K. Lee, M. N. Kim, B. M. Shin, Y. Uh, W. G. Lee, H. S. Lee, C. L. Chang, S. H. Kim, M. G. Shin, S. P. Sah, and D. W. Ryang.** 2007. Species distribution and susceptibility to azole antifungals of *Candida* bloodstream isolates from eight university hospitals in Korea. *Yonsei Med. J.* **48:**779–786.
122. **Leon, C., S. Ruiz-Santana, P. Saavedra, B. Almirante, J. Nolla-Salas, F. Alvarez-Leama, J. Garnacho-Montero, M. A. Leon, and the EPCAN Study Group.** 2006. A bedside scoring system ("*Candida* score") for early antifungal treatment in nonneutropenic critically ill patients with *Candida* colonization. *Crit. Care Med.* **43:**730–737.
123. **Levin, A. S., S. F. Costa, N. S. Mussi, M. Basso, S. I. Sinto, C. Machado, C. Geiger, M. C. Villares, A. Z. Schreiber, A. A. Barone, and M. L. Branchini.** 1998. *Candida parapsilosis* fungemia associated with implantable and semi-implantable central venous catheters and the hands of healthcare workers. *Diagn. Microbiol. Infect. Dis.* **30:**243–249.
124. **Levy, I., L. G. Rubin, S. Vasishtha, V. Tucci, and S. K. Sood.** 1998. Emergence of *Candida parapsilosis* as the predominant species causing candidemia in children. *Clin. Infect. Dis.* **26:**1086–1088.
125. **Levy, M. M., P. J. Pronvost, R. P. Dellinger, S. Townsend, R. K. Resar, T. P. Clemmer, and G. Ramsay.** 2004. Sepsis change bundles: converting guidelines into meaningful change in behavior and clinical outcome. *Crit. Care Med.* **32**(Suppl.)**:**S595–S597.
126. **Li, L., S. Redding, and A. Dongari-Bagtzoglou.** 2007. *Candida glabrata*, an emerging oral opportunistic pathogen. *J. Dent. Res.* **86:**204–215.
127. **Lin, M. Y., Y. Carmeli, J. Zumsteg, E. L. Flores, J. Tolentino, P. Sreeramoju, and S. G. Weber.** 2005. Prior antimicrobial therapy and risk for hospital-acquired *Candida glabrata* and *Candida krusei* fungemia: a case-case-control study. *Antimicrob. Agents Chemother.* **49:**4555–4560.
128. **Lockhart, S. R., S. Joly, K. Vargas, J. Swails-Wenger, L. Enger, and D. R. Soll.** 1999. Natural defenses against *Candida* colonization break down in the oral cavities of the elderly. *J. Dent. Res.* **78:**857–868.
129. **Lockhart, S. R., S. A. Messer, M. A. Pfaller, and D. J. Diekema.** 2008. Geographic distribution and antifungal susceptibility of the newly described species *Candida orthopsilosis* and *Candida metapsilosis* in comparison to the closely related species *Candida parapsilosis*. *J. Clin. Microbiol.* **46:**2659–2664.
130. **Lockhart, S. R., S. A. Messer, M. A. Pfaller, and D. J. Diekema.** 2009. Identification and susceptibility profile of *Candida fermentati* from a worldwide collection of *Candida guilliermondii* clinical isolates. *J. Clin. Microbiol.* **47:**242–244.
131. **Lockhart, S. R., S. A. Messer, M. Gherna, J. A. Bishop, W. G. Merz, M. A. Pfaller, and D. J. Diekema.** 2009. Identification of *Candida nivariensis* and *Candida bracarensis* in a large global collection of *Candida glabrata* isolates: comparison to the literature. *J. Clin. Microbiol.* **47:**1216–1217.
132. **Lockhart, S. R., N. Iqbal, K. A. Etienne, A. M. Ahlquist, M. Farley, W. Baughman, B. Siegel, L. H. Harrison, R. Hollick, S. A. Balajee, S. S. Magill, and T. Chiller.** 2009. Antifungal susceptibility of *Candida* isolates collected during population-based candidemia surveillance in Atlanta, GA, and Baltimore, MD, 2008, abstr. M-1927. *49th Intersci. Conf. Antimicrob. Agents Chemother.*
133. **Lockhart, S. R., D. J. Diekema, and M. A. Pfaller.** 2009. The epidemiology of fungal infections, p. 1–14. *In* E. J. Anaissie, M. R. McGinnis, and M. A. Pfaller (ed.), *Clinical Mycology*, 2nd ed. Elsevier Inc., Oxford, United Kingdom.
134. **Lupetti, A., A. Tavanti, P. Davini, E. Ghelardi, V. Corsini, I. Merusi, A. Boldrini, M. Campa, and S. Senesi.** 2002. Horizontal transmission of *Candida parapsilosis* candidemia in a neonatal intensive care unit. *J. Clin. Microbiol.* **40:**2363–2369.
135. **Luzzati, R., G. Amalfitano, L. Lazzarini, F. Soldori, S. Bellino, M. Solbeitic, M. C. Danzi, S. Vento, G. Todeschini, C. Vivenza, and E. Concia.** 2000. Nosocomial candidemia in non-neutropenic patients at an Italian

tertiary care hospital. *Eur. J. Clin. Microbiol. Infect. Dis.* **19:**602–607.

136. **MacArthur, R. D., M. Miller, T. Albertson, E. Panacek, D. Johnson, L. Teoh, and W. Barchuk.** 2004. Adequacy of early empiric antibiotic treatment and survival in severe sepsis: experience from the MONARCS trial. *Clin. Infect. Dis.* **38:**284–288.
137. **Magill, S. S., C. Shields, C. L. Spears, M. Choti, and W. G. Merz.** 2006. Triazole cross-resistance among *Candida* spp.: case report, occurrence among bloodstream isolates, and implications for antifungal therapy. *J. Clin. Microbiol.* **44:**529–535.
138. **Majoros, L., G. Kardos, A. Belok, A. Maraz, L. Asztalos, E. Csanky, Z. Barta, and B. Szabo.** 2003. Restriction enzyme analysis of ribosomal DNA shows that *Candida inconspicua* clinical isolates can be misidentified as *Candida norvegensis* with traditional diagnostic procedures. *J. Clin. Microbiol.* **41:**5250–5253.
139. **Majoros, L., G. Kardos, B. Szabo, M. Kovacs, and A. Maraz.** 2005. Fluconazole susceptibility testing of *Candida inconspicua* clinical isolates: comparison of four methods. *J. Antimicrob. Chemother.* **55:**275–276.
140. **Majoros, L., G. Kardos, P. Feisgt, and B. Szabo.** 2005. Efficacy of amphotericin B and flucytosine against fluconazole-resistant *Candida inconspicua* clinical isolates. *J. Antimicrob. Chemother.* **56:**253–254.
141. **Majoros, L., G. Kardos, B. Szabo, and M. Sipiczki.** 2005. Caspofungin susceptibility testing of *Candida inconspicua*: correlation of different methods with the minimal fungicidal concentration. *Antimicrob. Agents Chemother.* **49:**3486–3488.
142. **Malani, A., J. Hmoud, L. Chiu, P. L. Carver, A. Bielaczc, and C. A. Kauffman.** 2005. *Candida glabrata* fungemia: experience in a tertiary care center. *Clin. Infect. Dis.* **41:**975–981.
143. **Manzoni, P., I. Stolfi, L. Pugni, L. Decembrino, C. Magnani, G. Vetrano, E. Tridapalli, G. Corona, C. Giovannozzi, D. Farina, R. Arisio, F. Merletti, M. Maule, F. Mosca, R. Pedicino, M. Stronati, M. Mostert, and G. Gomirato for the Italian Task Force for the Study and Prevention of Neonatal Fungal Infections and the Italian Society of Neonatology.** 2007. A multicenter, randomized trial of prophylactic fluconazole in preterm neonates. *N. Engl. J. Med.* **356:**2483–2495.
144. **Marchetti, O., J. Bille, U. Fluckinger, P. Eggimann, C. Ruef, J. Garbino, T. Calandra, M. P. Glauser, M. G. Tauber, and D. Pittet.** 2004. Epidemiology of candidemia in Swiss tertiary care hospitals: secular trends 1991–2000. *Clin. Infect. Dis.* **38:**311–320.
145. **Marco, F., S. R. Lockhart, M. A. Pfaller, C. Pujol, M. S. Rangel-Frausto, T. Wiblin, H. M. Blumberg, J. E. Edwards, W. Jarvis, L. Saiman, J. E. Patterson, M. G. Rinaldi, R. P. Wenzel, and D. R. Soll.** 1999. Elucidating the origins of nosocomial infections with *Candida albicans* by DNA fingerprinting with the complex probe Ca3. *J. Clin. Microbiol.* **37:**2817–2828.
146. **Mardani, M., H. A. Hanna, E. Girgawy, and I. Raad.** 2000. Nosocomial *Candida guilliermondii* fungemia in cancer patients. *Infect. Control Hosp. Epidemiol.* **21:**336–337.
147. **Marichal, P., H. Vanden Bossche, F. C. Odds, G. Nobels, D. W. Warnock, V. Timmerman, C. Van Broeckhoven, S. Fay, and P. Mose-Larsen.** 1997. Molecular biological characterization of an azole-resistant *Candida glabrata* isolate. *Antimicrob. Agents Chemother.* **41:**2229–2237.
148. **Marr, K. A., K. Seidel, T. C. White, and R. A. Bowden.** 2000. Candidemia in allogeneic blood and marrow transplant recipients: evolution of risk factors after the adoption of prophylactic fluconazole. *J. Infect. Dis.* **181:** 309–316.
149. **Masala, L., R. Luzzati, J. Maccacaro, L. Antozzi, E. Concia, and R. Fontana.** 2003. Nosocomial cluster of *Candida guilliermondii* fungemia in surgical patients. *Eur. J. Clin. Microbiol. Infect. Dis.* **22:**686–688.
150. **McClenny, N. B., H. Fei, E. J. Baron, A. C. Gales, A. Houston, R. J. Hollis, and M. A. Pfaller.** 2002. Change in colony morphology of *Candida lusitaniae* in association with development of amphotericin B resistance. *Antimicrob. Agents Chemother.* **46:**1325–1328.
151. **Mean, M., O. Marchetti, and T. Calandra.** 2008. Bench-to-bedside review: *Candida* infections in the intensive care unit. *Crit. Care* **12:**204–212.
152. **Medeiros, E. A. S., T. J. Lott, A. L. Colombo, P. Godoy, A. P. Coutinho, M. S. Broga, M. Nucci, and M. E. Brandt.** 2007. Evidence for a pseudo-outbreak of *Candida guilliermondii* fungemia in a university hospital in Brazil. *J. Clin. Micrbiol.* **45:**942–947.
153. **Meis, J. F. G. M., M. Ruhnke, B. E. DePauw, F. C. Odds, W. Siegert, and P. E. Verweij.** 1999. *Candida dubliniensis* candidemia in patients with chemotherapy-induced neutropenia and bone marrow transplantation. *Emerg. Infect. Dis.* **5:**150–153.
154. **Merz, W. G.** 1984. *Candida lusitaniae*: frequency of recovery, colonization, infection and amphotericin B resistance. *J. Clin. Microbiol.* **20:**1194–1195.
155. **Merz, W. G., J. E. Karp, D. Schron, and R. Saral.** 1986. Increased incidence of fungemia caused by *Candida krusei*. *J. Clin. Microbiol.* **24:**581–584.

155a. **Miller, L. G., R. A. Hajjeh, and J. E. Edwards, Jr.** 2001. Estimating the cost of nosocomial candidemia in the United States. *Clin. Infect. Dis.* **32:**1110.

156. **Miller, N. S., J. D. Dick, and W. G. Merz.** 2006. Phenotypic switching in *Candida lusitaniae* on copper sulfate indicator agar: association with amphotericin B resistance and filamentation. *J. Clin. Microbiol.* **44:** 1536–1539.
157. **Minari, A., R. Hachem, and I. Raad.** 2001. *Candida lusitaniae*: a cause of breakthrough fungemia in cancer patients. *Clin. Infect. Dis.* **32:**186–190.
158. **Mok, W. Y., and M .S. Barreto da Silva.** 1984. Mycoflora of human dental surfaces. *Can. J. Microbiol.* **30:**1205–1209.
159. **Mokaddas, E. M., N. A. Al-Sweih, and Z. U. Khan.** 2007. Species distribution and antifungal susceptibility of *Candida* bloodstream isolates in Kuwait: a 10-year study. *J. Med. Microbiol.* **56:**255–259.
160. **Moran, C., C. A. Grussemeyer, J. R. Spalding, D. K. Benjamin, Jr., and S. D. Reed.** 2009. *Candida albicans* and non-*albicans* bloodstream infections in adult and pediatric patients: comparison of mortality and costs. *Pediatr. Infect. Dis. J.* **28:**433–435.
161. **Morgan, J.** 2005. Global trends in candidemia: review of reports from 1995–2005. *Curr. Infect. Dis. Rep.* **7:** 429–439.
162. **Morgan, J., M. I. Meltzer, B. D. Plikaytis, A. N. Sofair, S. Huie-White, S. Wilcox, L. H. Harrison, E. C. Seaberg, R. A. Hajjeh, and S. M. Teutsch.** 2005. Excess mortality, hospital stay, and cost due to candidemia: a case-control study using data from population-based candidemia surveillance. *Infect. Control Hosp. Edpidemiol.* **26:**540–547.
163. **Morgan, P., D. Sanglard, M. Donnelly, B. Shanley, J. Sullivan, and C. Coleman.** 1998. Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. *Antimicrob. Agents Chemother.* **42:**1819–1830.

164. **Morrell, M., V. J. Fraser, and M. I. Kollef.** 2005. Delaying empiric treatment of *Candida* bloodstream infection until positive blood culture results are obtained: a potential risk factor for mortality. *Antimicrob. Agents Chemother.* **49:**3640–3645.
165. **Munoz, P., A. Burillo, and E. Bouza.** 2000. Criteria used when initiating antifungal therapy against *Candida* spp. in the intensive care unit. *Int. J. Antimicrob. Agents* **15:** 83–90.
166. **Neofytos, D., D. Horn, E. Anaissie, W. Steinback, A. Olyaei, J. Fishman, M. Pfaller, C. Cheng, K. Webster, and K. Marr.** 2009. Epidemiology and outcome of invasive fungal infection in adult hematopoietic stem cell transplant recipients: analysis of multicenter PATH Alliance Registry. *Clin. Infect. Dis.* **48:**265–273.
167. **Neofytos, D., J. A. Fishman, D. Horn, E. Anaissie, C. H. Chang, A. Olyaei, M. Pfaller, W. J. Steinbach, K. M. Webster, and K. A. Marr.** 2010. Epidemiology and outcome of invasive fungal infections in solid organ transplant recipients. *Transpl. Infect. Dis.* **12:**220–229.
168. **Nguyen, M. H., J. E. Peacock, Jr., A. J. Morris, D. C. Tanner, M. L. Nguyen, D. R. Snydman, M. M. Wagener, M. G. Rinaldi, and V. L. Yu.** 1996. The changing face of candidemia: emergence of non-*Candida albicans* species and antifungal resistance. *Am. J. Med.* **100:**617–623.
169. **Nguyen, M. H., A. J. Morris, M. E. Dobson, D. R. Snydman, J .E. Peacock, Jr., M. G. Rinaldi, and V. L. Yu.** 1996. *Candida lusitaniae*: an important emerging cause of candidemia. *Infect. Dis. Clin. Pract.* **5:**273–278.
170. **Nho, S., M. J. Anderson, C. B. Moore, and D. W. Denning.** 1997. Species differentiation by internally transcribed spacer PCR and *Hha*I digestion of fluconazole-resistant *Candida krusei*, *Candida inconspicua*, and *Candida norvegensis* strains. *J. Clin. Microbiol.* **35:**1036–1039.
171. **Nielsen, H., J. Stenderup, B. Bruun, and J. Ladefoged.** 1990. *Candida norvegensis* peritonitis and invasive disease in a patient on continuous ambulatory peritoneal dialysis. *J. Clin. Microbiol.* **28:**1664–1665.
172. **Nielsen, H., and J. Stenderup.** 1996. Invasive *Candida norvegensis* infection in immunocompromised patients. *Scand. J. Infect. Dis.* **28:**311–312.
173. **Nucci, M., and K. A. Marr.** 2005. Emerging fungal diseases. *Clin. Infect. Dis.* **41:**521–526.
174. **Odds, F. C., M. F. Hanson, A. D. Davidson, M. D. Jacobsen, P. Wright, J. A. Whyte, N. A. R. Gow, and B. L. Jones.** 2007. One year prospective survey of *Candida* bloodstream infections in Scotland. *J. Med. Microbiol.* **56:**1066–1075.
175. **Oliviera, S. C. D., C. Pina-Vaz, D. Mendonca, and A. G. Rodrigues.** 2008. A first Portuguese epidemiological survey of fungemia in a university hospital. *Eur. J. Clin. Microbiol. Infect. Dis.* **27:**365–374.
176. **Ostrosky-Zeichner, L.** 2003. New approaches to the risk of *Candida* in the intensive care unit. *Curr. Opin. Infect. Dis.* **16:**533–537.
177. **Ostrosky-Zeichner, L.** 2004. Prophylaxis and treatment of invasive candidiasis in the intensive care setting. *Eur. J. Clin. Microbiol. Infect. Dis.* **23:**739–744.
178. **Ostrosky-Zeichner, L., and P. G. Pappas.** 2006. Invasive candidiasis in the intensive care unit. *Crit. Care Med.* **34:**857–863.
179. **Ostrosky-Zeichner, L., P. G. Pappas, S. Shoham, A. Reboli, M. A. Barron, C. Sims, C. Wood, and J. D. Sobel.** 2011. Improvement of a clinical prediction role for clinical trials on prophylaxis for invasive candidiasis in the intensive care unit. *Mycoses* **54:**46–51.
180. **Panackal, A. A., J. L. Gribskov, J. F. Staab, K. A. Kirby, M. Rinalid, and K. A. Marr.** 2006. Clinical significance of azole antifungal drug cross-resistance in *Candida glabrata*. *J. Clin. Microbiol.* **44:**1740–1743.
181. **Panizo, M. M., V. Reviakina, M. Dolande, and S. Selgrad.** 2009. *Candida* spp. in vitro susceptibility profile to four antifungal agents. Resistance surveillance study in Venezuelan strains. *Med. Mycol.* **47:**137–143.
182. **Paphitou, N. I., L. Ostrosky-Zeichner, and J. H. Rex.** 2005. Rules of identifying patients at increased risk for candidal infections in the surgical intensive care unit: approach to developing practical criteria for systematic use in antifungal prophylaxis trials. *Med. Mycol.* **43:**235–243.
183. **Pappagianis, H. D., M. S. Collins, R. Hector, and J. Remington.** 1979. Development of resistance to amphotericin B in *Candida lusitaniae* infecting a human. *Antimicrob. Agents Chemother.* **16:**123–126.
184. **Pappas, P. G.** 2004. Invasive candidiasis. *Infect. Dis. Clin. N. Am.* **20:**485–506.
185. **Pappas, P. G., J. H. Rex, J. Lee, R. J. Hamill, R. A. Larsen, W. Powderly, C. A. Kauffman, N. Hyslop, J. E. Mangino, S. Chapman, H. W. Horowitz, J. E. Edwards, W. E. Dismukes, and the NIAID Mycoses Study Group.** 2003. A prospective observational study of candidemia: epidemiology, therapy, and influences on mortality in hospitalized adult and pediatric patients. *Clin. Infect. Dis.* **37:**634–643.
186. **Pappas, P. G., J. H. Rex, J. D. Sobel, S. G. Filler, W. E. Dismukes, T. J. Walsh, and J. E. Edwards for the Infectious Diseases Society of America.** 2004. Guidelines for the treatment of candidiasis. *Clin. Infect. Dis.* **38:**161–189.
187. **Pappas, P. G., C. A. Kauffman, D. Andes, D. K. Benjamin, Jr., T. F. Calandra, J. E. Edwards, Jr., S. G. Filler, J. F. Fisher, B. J. Kullberg, L. Ostrosky-Zeichner, A. C. Reboli, J. H. Rex, T. J. Walsh, and J. D. Sobel for the Infectious Diseases Society of America.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48:**503–535.
188. **Park, B. J., B. A. Arthington-Skaggs, R. A. Hajjeh, N. Iqbal, M. A. Ciblak, W. Lee-Yang, M. E. Hairston, M. Phelan, B. D. Plikaytis, A. N. Sofair, L. H. Harrison, S. K. Fridkin, and D. W. Warnock.** 2006. Evaluation of amphotericin B interpretive breakpoints for *Candida* bloodstream infections by correlation with therapeutic outcomes. *Antimicrob. Agents Chemother.* **50:**1287–1292.
189. **Parkins, M. D., D. M. Sabuda, S. Elsayed, and K. B. Laupland.** 2007. Adequacy of empirical antifungal therapy and effect on outcome among patients with invasive *Candida* species infection. *J. Antimicrob. Chemother.* **60:** 613–618.
190. **Pasqualotto, A. C., A. G. Antunes, and L. C. Severo.** 2006. *Candida guilliermondii* as the aetiology of candidosis. *Rev. Inst. Med. Trop. São Paulo* **48:**123–127.
191. **Pasqualotto, A. C., R. A. Zimmerman, S. H. Alves, V. R. Aquino, D. Branco, D. Wittgen, A. do Amarol, R. Cechinel, S. M. Colores, I. G. da Rocha, L. C. Severo, and T. C. Sukiennik.** 2008. Take control over your fluconazole prescriptions: the growing important of *Candida glabrata* as an agent of candidemia in Brazil. *Infect. Control Hosp. Epidemiol.* **29:**898–899.
192. **Pastor, P., F. G. Medley, and T. V. Murphy.** 2000. Meningococcal disease in Dallas County, Texas: results of a six-year population-based study. *Pediatr. Infect. Dis.* **19:**324–328.
193. **Patel, M., D. F. Kunz, V. M. Trivedi, M. G. Jones, S. A. Moser, and J. W. Baddley.** 2005. Initial management

of candidemia at an academic medical center: evaluation of the IDSA guidelines. *Diagn. Microbiol. Infect. Dis.* **52:**29–34.

194. **Pelz, R. K., P. A. Lipset, S. M. Swoboda, M. Diener-West, N. R. Power, R. G. Brower, T. M. Perl, J. M. Hammond, and C. W. Hendrix.** 2000. *Candida* infections: outcomes and attributable ICU costs in critically ill patients. *J. Intensive Care Med.* **15:**255–261.
195. **Pelz, R. K., C. W. Hendrix, S. M. Swoboda, M. Diener-West, W. G. Merz, J. Hammond, and P. A. Lipsett.** 2001. Double-blind placebo-controlled trial of fluconazole to prevent candidal infections in critically ill surgical patients. *Ann. Surg.* **233:**542–548.
196. **Peman, J., E. Canton, M. Gobernado, and the Spanish Working Group ECMM on Candidaemia.** 2005. Epidemiology and antifungal susceptibility of *Candida* species isolated from blood: results of a 2-year multicentre study in Spain. *Eur. J. Clin. Microbiol. Infect. Dis.* **24:**23–30.
197. **Peman, J., M. Bosch, E. Canton, A. Viades, I. Jarque, M. Gomez-Garcia, J. M. Garcia-Martinez, and M. Gobernado.** 2008. Fungemia due to *Candida guilliermondii* in a pediatric and adult population during a 12-year period. *Diagn. Microbiol. Infect. Dis.* **60:**109–112.
198. **Perlin, D. S.** 2007. Resistance to echinocandins-class antifungal drugs. *Drug Resist. Updates* **10:**121–130.
199. **Perlroth, J., B. Choi, and B. Spellberg.** 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. *Med. Mycol.* **45:**321–346.
200. **Pfaller, M. A.** 1996. Nosocomial candidiasis: emerging species, reservoirs, and modes of transmission. *Clin. Infect. Dis.* **22**(Suppl. 2)**:**S89–S94.
201. **Pfaller, M. A., I. Cabezudo, F. Koontz, M. Bale, and R. Gingrich.** 1987. Predictive value of surveillance cultures for systemic infection due to *Candida* species. *Eur. J. Clin. Microbiol.* **6:**628–633.
202. **Pfaller, M. A., S. A. Messer, and R. J. Hollis.** 1994. Strain delineation and antifungal susceptibilities of epidemiologically related and unrelated isolates of *Candida lusitaniae*. *Diagn. Microbiol. Infect. Dis.* **20:**127–133.
203. **Pfaller, M. A., S. A. Messer, and A. Bolstrom.** 1998. Evaluation of Etest for determining in vitro susceptibility of yeast isolates to amphotericin B. *Diagn. Microbiol. Infect. Dis.* **32:**233–237.
204. **Pfaller, M. A., S. R. Lockhart, C. Pujol, J. A. Swails-Wenger, S. A. Messer, M. B. Edmond, R. N. Jones, R. P. Wenzel, and D. R. Soll.** 1998. Hospital specificity, region specificity, and fluconazole resistance of *Candida albicans* bloodstream isolates. *J. Clin. Microbiol.* **36:** 1518–1529.
205. **Pfaller, M. A., S. A. Messer, S. Gee, S. Joly, C. Pujol, D. J. Sullivan, D. C. Coleman, and D. R. Soll.** 1999. In vitro susceptibility of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. *J. Clin. Microbiol.* **37:**870–872.
206. **Pfaller, M. A., and D. J. Diekema.** 2002. Role of sentinel surveillance of candidemia: trends in species distribution and antifungal susceptibility. *J. Clin. Microbiol.* **40:** 3551–3557.
207. **Pfaller, M. A., D. J. Diekema, R. N. Jones, S. A. Messer, R. J. Hollis, and the SENTRY Participants Group.** 2002. Trends in antifungal susceptibility of *Candida* spp. isolated from pediatric and adult patients with bloodstream infections: SENTRY Antimicrobial Surveillance Program, 1997 to 2000. *J. Clin. Microbiol.* **40:**852–856.
208. **Pfaller, M. A., S. A. Messer, L. Boyken, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2003. Variation in susceptibility of bloodstream isolates of *Candida glabrata* to fluconazole according to patient age and geographic location. *J. Clin. Microbiol.* **41:**2176–2179.
209. **Pfaller, M. A., S. A. Messer, L. Boyken, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2004. Geographic variation in the susceptibility of invasive isolates of *Candida glabrata* to seven systemically active antifungal agents: a global assessment from the ARTEMIS Antifungal Surveillance Program conducted in 2001 and 2002. *J. Clin. Microbiol.* **42:**3142–3146.
210. **Pfaller, M. A., and D. J. Diekema.** 2004. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J. Clin. Microbiol.* **42:**4419–4431.
211. **Pfaller, M. A., and D. J. Diekema.** 2004. Twelve years of fluconazole susceptibility of bloodstream isolates of *Candida*. *Clin. Microbiol. Infect.* **10**(Suppl. 1)**:**11–23.
212. **Pfaller, M. A., L. Boyken, S. A. Messer, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2004. Evaluation of the Etest method using Mueller-Hinton agar with glucose and methylene blue for determining amphotericin B MICs for 4,936 clinical isolates of *Candida* species. *J. Clin. Microbiol.* **42:**4977–4979.
213. **Pfaller, M. A., S. A. Messer, L. Boyken, R. J. Hollis, C. Rice, S. Tendolkar, and D. J. Diekema.** 2004. In vitro activities of voriconazole, posaconazole, and fluconazole against 4,169 clinical isolates of *Candida* spp. and *Cryptococcus neoformans* collected during 2001 and 2002 in the ARTEMIS Global Antifungal Surveillance Program. *Diagn. Microbiol. Infect. Dis.* **48:**201–205.
214. **Pfaller, M. A., D. J. Diekema, A. L. Colombo, C. Kibbler, K. Peng, D. L. Gibbs, V. A. Newell, and the Global Antifungal Surveillance Group.** 2006. *Candida rugosa*, an emerging pathogen with resistance to azoles: geographic and temporal trends from the ARTEMIS DISK AntifungalSurveillanceProgram.*J.Clin.Microbiol.***44:**3578–3582.
215. **Pfaller, M. A., D. J. Diekema, M. Mendez, C. Kibbler, P. Erzebert, S. C. Chang, D. L. Gibbs, V. A. Newell, and the Global Antifungal Surveillance Group.** 2006. *Candida guilliermondii*, an opportunistic pathogen with decreased susceptibility to fluconazole: geographic and temporal trends from the ARTEMIS DISK Antifungal Susceptibility Program. *J. Clin. Microbiol.* **44:**3551–3556.
216. **Pfaller, M. A., P. G. Pappas, and J. R. Wingard.** 2006. Invasive fungal pathogens: current epidemiological trends. *Clin. Infect. Dis.* **43:**S3–S14.
217. **Pfaller, M. A., L. Boyken, R. J. Hollis, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2006. In vitro susceptibilities of *Candida* spp. to caspofungin: four years of global surveillance. *J. Clin. Microbiol.* **44:**760–763.
218. **Pfaller, M. A., and D. J. Diekema.** 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin. Microbiol. Rev.* **20:**133–163.
219. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, J. F. Meis, I. M. Gould, W. Fu, A. L. Colombo, E. Rodriguez-Noriega, and the Global Antifungal Surveillance Study.** 2007. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2005: an 8.5 year analysis of susceptibilities of *Candida* species and other yeast species to fluconazole and voriconazole determined by CLSI standardized disk diffusion testing. *J. Clin. Microbiol.* **45:**1735–1745.
220. **Pfaller, M. A., L. Boyken, R. J. Hollis, J. Kroeger, S. A. Messer, S. Tendolkar, and D. J. Diekema.** 2008. In vitro susceptibility of invasive isolates of *Candida* spp. to anidulafungin, caspofungin, and micafungin: six years of global surveillance. *J. Clin. Microbiol.* **46:**150–156.

221. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, K. P. Ng, A. Colombo, J. Finquelievich, R. Barnes, J. Wadula, and the Global Antifungal Surveillance Group.** 2008. Geographic and temporal trends in isolation and antifungal susceptibility of *Candida parapsilosis*: a global assessment from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *J. Clin. Microbiol.* **46:**842–849.
222. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, E. Nagy, S. Dobiasova, M. Rinaldi, R. Barton, A. Veselov, and the Global Antifungal Surveillance Group.** 2008. *Candida krusei*, a multidrug-resistant opportunistic fungal pathogen: geographic and temporal trends from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. *J. Clin. Microbiol.* **46:**515–521.
223. **Pfaller, M. A., S. A. Messer, L. Boyken, S. Tendolkar, R. J. Hollis, and D. J. Diekema.** 2008. Selection of a surrogate agent (fluconazole or voriconazole) for initial susceptibility testing of posaconazole against *Candida* spp.: results from a global antifungal surveillance program. *J. Clin. Microbiol.* **46:**551–559.
224. **Pfaller, M. A., D. J. Diekema, L. Ostrosky-Zeichner, J. H. Rex, B. D. Alexander, D. Andes, S. D. Brown, V. Chaturvedi, M. A. Ghannoum, C. C. Knapp, D. J. Sheehan, and T. J. Walsh.** 2008. Correlation of MIC with outcome for *Candida* species tested against caspofungin, anidulafungin, and micafungin: analysis and proposal for interpretive MIC breakpoints. *J. Clin. Microbiol.* **46:**2620–2629.
225. **Pfaller, M. A., S. A. Messer, R. J. Hollis, L. Boyken, S. Tendolkar, J. Kroeger, and D. J. Diekema.** 2009. Variation in susceptibility of bloodstream isolates of *Candida glabrata* to fluconazole according to patient age and geographic location in the United States in 2001 to 2007. *J. Clin. Microbiol.* **47:**3185–3190.
226. **Pfaller, M. A., L. Boyken, R. J. Hollis, J. Kroeger, S. A. Messer, S. Tendolkar, R. N. Jones, J. Turnidge, and D. J. Diekema.** 2010. Wild-type MIC distributions and epidemiological cutoff values for the echinocandins and *Candida* spp. *J. Clin. Microbiol.* **48:**52–56.
227. **Pfaller, M. A., and D. J. Diekema.** 2010. Epidemiology of invasive mycoses in North America. *Crit. Rev. Microbiol.* **36:**1–53.
228. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, R. Barton, H. Bijie, J. Bille, S. C. Chang, M. da Luz Martens, A. Duse, D. Dzieranowski, D. Ellis, J. Finquelievich, I. Gould, D. Gur, A. Hoosen, K. Lee, N. Malatora, M. Mallie, N. K. Peng, G. Petrikos, A. Santiago, J. Trupl, A. M. Vanden Abeele, J. Wadula, M. Zaidi, and the Global Antifungal Surveillance Group.** 2010. Geographic variation in the frequency of isolation and fluconazole and voriconazole susceptibilities of *Candida glabrata*: an assessment from the ARTEMIS DISK Global Antifungal Surveillance Program. *Diagn. Microbiol. Infect. Dis.* **67:**162–171.
229. **Pfaller, M. A., D. J. Diekema, D. L. Gibbs, V. A. Newell, D. Ellis, V. Tullio, A. Rodloff, W. Fu, T. A. Ling, and the Global Antifungal Surveillance Group.** 2010. Results from the ARTEMIS DISK Global Antifungal Surveillance Study, 1997 to 2007: a 10.5-year analysis of susceptibilities of *Candida* species to fluconazole and voriconazole determined by CLSI standardized disk diffusion. *J. Clin. Microbiol.* **48:**1366–1377.
230. **Piarroux, R., F. Grenouillet, P. Balvay, V. Tran, G. Blasoc, L. Millon, and A. Boillot.** 2004. Assessment of preemptive treatment to prevent severe candidiasis in critically ill surgical patients. *Crit. Care Med.* **32:**2443–2449.
231. **Pittet, D., N. Li, R. F. Woolson, and R. P. Wenzel.** 1997. Microbiological factors influencing the outcome of nosocomial bloodstream infections: a 6-year validated, population-based model. *Clin. Infect. Dis.* **24:**1068–1078.
232. **Poikonen, E., O. Lyytikaninen, V. J. Anttila, and P. Ruutu.** 2003. Candidemia in Finland, 1995–1999. *Emerg. Infect. Dis.* **9:**985–990.
233. **Prentice, H. G., C. C. Kibbler, and A. G. Prentice.** 2000. Towards a targeted, risk-based, antifungal strategy in neutropenic patients. *Br. J. Haematol.* **110:**273–284.
234. **Procop, G. W., and G. D. Roberts.** 2004. Emerging fungal diseases: the importance of the host. *Clin. Lab. Med.* **24:**691–719.
235. **Puzniak, L., S. Teutsch, W. Powderly, and L. Polish.** 2004. Has the epidemiology of nosocomial candidemia changed? *Infect. Control Hosp. Epidemiol.* **25:**628–633.
236. **Qi, Q. G., T. Hu, and X. O. Zhou.** 2005. Frequency, species and molecular characterization of oral *Candida* in hosts of different age in China. *J. Oral Pathol. Med.* **34:**352–356.
237. **Ramage, G., E. Mowat, B. Jones, C. Williams, and J. Lopez-Ribot.** 2009. Our current understanding of fungal biofilms. *Crit. Rev. Microbiol.* **35:**340–355.
238. **Redding, S. W., W. R. Kirkpatrick, B. J. Coco, L. Sadkowski, A. W. Fothergill, M. G. Rinaldi, T. Y. Eng, and T. F. Patterson.** 2002. *Candida glabrata* oropharyngeal candidiasis in patients receiving medication treatment for head and neck cancer. *J. Clin. Microbiol.* **40:**1879–1881.
239. **Redding, S. W., W. R. Kirkpatrick, S. Sarille, B. J. Coco, W. White, A. Fothergill, M. Rinaldi, T. Eng, T. F. Patterson, and J. Lopez-Ribot.** 2003. Multiple patterns of resistance to fluconazole in *Candida glabrata* isolates from a patient with oropharyngeal candidiasis receiving head and neck radiation. *J. Clin. Microbiol.* **41:**619–622.
240. **Reinhardt, J. F., R. P .J. Ruane, L .J. Walker, and W. L. George.** 1985. Intravenous catheter-associated fungemia due to *Candida rugosa*. *J. Clin. Microbiol.* **22:**1056–1057.
241. **Reiss, E., B. A. Lasker, N. J. Iqbal, M. J. James, and B. A. Arthington-Skaggs.** 2008. Molecular epidemiology of *Candida parapsilosis* sepsis from outbreak investigations in neonatal intensive care units. *Infect. Genet. Evol.* **8:**103–109.
242. **Rentz, A. M., M. T. Halpern, and R. Bowden.** 1998. Impact of candidemia on length of hospital stay, outcome, and overall cost of illness. *Clin. Infect. Dis.* **27:**781–788.
243. **Rex, J. H.** 2006. Antifungal prophylaxis in the intensive care unit: who should get it? *Crit. Care Med.* **34:**1286–1287.
244. **Rex, J. H., and M. A. Pfaller.** 2002. Has antifungal susceptibility testing come of age? *Clin. Infect. Dis.* **35:**982–989.
245. **Richardson, M., and C. Lass-Florl.** 2008. Changing epidemiology of systemic fungal infections. *Clin. Microbiol. Infect.* **14**(Suppl. 4)**:**5–24.
246. **Richet, H., P. Roux, C. Des Champs, Y. Esnault, A. Andremont, and the French Candidemia Study Group.** 2002. Candidemia in French hospitals: incidence rates and characteristics. *Clin. Microbiol. Infect.* **8:**405–412.
247. **Riddell, J., IV, and C. A. Kauffman.** 2008. The evolution of resistant *Candida* species in cancer centers: implications for treatment and prophylaxis. *Cancer* **112:**2334–2337.
248. **Rivers, E., B. Nguyen, S. Havstad, J. Ressler, A. Muzzin, B. Knoblich, E. Peterson, and M. Tomlanovich for the Early Goal-Directed Therapy Collaborative Group.** 2001. Early goal-directed therapy in the treatment of severe sepsis and septic shock. *N. Engl. J. Med.* **345:**1368–1377.

249. **Ruan, S. Y., L. N. Lee, J. S. Jerng, C. J. Yu, and P. R. Hsueh.** 2008. *Candida glabrata* fungemia in intensive care units. *Clin. Microbiol. Infect.* **14:**136–140.
250. **Ruan, S. Y., and P. R. Hsueh.** 2009. Invasive candidiasis: an overview from Taiwan. *J. Formos. Med. Assoc.* **108:** 443–451.
251. **Sabatelli, F., R. Patel, P. A. Mann, C. A. Mendrick, C. C. Norris, R. Hare, D. Loebenberg, T. A. Black, and P. M. McNicholas.** 2006. In vitro activity of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. *Antimicrob. Agents Chemother.* **50:**2009–2015.
252. **Safdar, A., D. S. Perkins, and D. Armstrong.** 2002. Hematogenous infections due to *Candida parapsilosis*: changing trends in fungemic patients at a comprehensive cancer center during the last four decades. *Diagn. Microbiol. Infect. Dis.* **44:**11–16.
253. **Saiman, L., E. Ludington, M. Pfaller, S. Rangel-Frausto, R. T. Wiblin, J. Dawson, H. M. Blumberg, J. E. Patterson, M. Rinaldi, J. E. Edwards, R. P. Wenzel, and W. Jarvis.** 2000. Risk factors for candidemia in neonatal intensive care unit patients. *Pediatr. Infect. Dis. J.* **19:**319–324.
254. **Sanchez, V., J. A. Vazquez, D. Barth-Jones, L. Dembry, J. D. Sobel, and M. J. Zervos.** 1992. Epidemiology of nosocomial acquisition of *Candida lusitaniae*. *J. Clin. Microbiol.* **30:**3005–3008.
255. **Sandven, P., K. Nilsen, A. Digranes, T. Tjade, and J. Lassen.** 1997. *Candida norvegensis*: a fluconazole-resistant species. *Antimicrob. Agents Chemother.* **41:**1375–1376.
256. **Sandven, P., L. Beranger, A. Digranes, H. H. Hawkland, T. Mannsaker, P. Gausted, and the Norwegian Yeast Study Group.** 2006. Candidemia in Norway (1991 to 2003): results from a nationwide study. *J. Clin. Microbiol.* **44:**1977–1981.
257. **Sanford, G. R., W. G. Merz, J. R. Wingard, P. Charache, and R. Saral.** 1980. The value of fungal surveillance cultures as predictors of systemic fungal infections. *J. Infect. Dis.* **142:**503–509.
258. **Sanglard, D., F. Ischer, D. Calabrese, P. A. Majcherczyk, and J. Bille.** 1999. The ATP binding cassette transporter gene *CgCDR1* from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. *Antimicrob. Agents Chemother.* **43:**2753–2765.
259. **Sanguinetti, M., B. Posterara, B. Flori, S. Ranno, R. Torell, and G. Fadda.** 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. *Antimicrob. Agents Chemother.* **49:**668–679.
260. **San Miguel, L. G., J. Cobo, E. Otheo, A. Sanchez-Sousa, V. Abraira, and S. Mueno.** 2005. Secular trends of candidemia in a large tertiary-care hospital from 1988 to 2000: emergence of *Candida parapsilosis*. *Infect. Control Hosp. Epidemiol.* **26:**548–552.
261. **San Millan, R. M., L. C. Wu, I. F. Salkin, and P. F. Lehmann.** 1997. Clinical isolates of *Candida guilliermondii* include *Candida fermentati*. *Int. J. Syst. Bacteriol.* **47:**385–393.
262. **Sarvikivi, E., O. Lyytikalnen, D. R. Soll, C. Pujol, M. A. Pfaller, M. Richardson, P. Koukila-Kahklola, P. Luukkainen, and H. Saxen.** 2005. Emergence of fluconazole resistance in a *Candida parapsilosis* strain that caused infections in a neonatal intensive care unit. *J. Clin. Microbiol.* **43:**2729–2735.
263. **Saxen, H., M. Virtanen, P. Carlson, K. Hoppu, M. Pohjavuori, M. Vaara, J. Voupio-Varkila, and H. Peltola.** 1995. Neonatal *Candida parapsilosis* outbreak with a high case fatality rate. *Pediatr. Infect. Dis.* **14:**776–789.
264. **Sendid, B., A. Cotteau, N. Francois, A. D'Haveloose, A. Standaert, D. Camus, and D. Poulain.** 2006. Candidemia and antifungal therapy in a French university hospital: rough trends over a decade and possible links. *BMC Infect. Dis.* **6:**80–89.
265. **Shorr, A. F., D. R. Lazarus, J. H. Sherner, W. L. Jackson, M. Morrel, V. J. Fraser, and M. H. Kollef.** 2007. Do clinical features allow for accurate predictions of fungal pathogens in bloodstream infections? Potential implications of the increasing prevalence of non-*albicans* candidemia. *Crit. Care Med.* **35:**1077–1083.
266. **Shorr, A. F., V. Gupta, X. Sun, R. S. Johannes, J. Spalding, and Y. P. Tabak.** 2009. Burden of early-onset candidemia: analysis of culture-positive bloodstream infections from a large U.S. database. *Crit. Care Med.* **37:**2519–2526.
267. **Sipsas, N. V., R. E. Lewis, J. Tarrand, R. Hochem, K. V. Rolston, I. I. Raad, and D. P. Kontoyiannis.** 2009. Candidemia in patients with hematologic malignancies in the era of new antifungal agents (2001–2007): stable incidence but changing epidemiology of a still frequently lethal infection. *Cancer* **115:**4745–4752.
268. **Smith, P. B., J. Morgan, D. K. Benjamin, S. K. Fridkin, L. T. Sanza, L. H. Harrison, A. M. Sofair, S. Huie-White, and D. K. Benjamin, Jr.** 2007. Excess cost of hospital care associated with neonatal candidemia. *Pediatr. Infect. Dis. J.* **26:**197–200.
269. **Sobel, J. D.** 2007. The emergence of non-*albicans Candida* species as causes of invasive candidiasis and candidemia. *Curr. Fungal Infect. Rep.* **1:**42–48.
270. **Sobel, J. D., and J. H. Rex.** 2001. Invasive candidiasis: turning risk into a practical prevention policy. *Clin. Infect. Dis.* **33:**187–190.
271. **Sofair, A. N., G. M. Lyon, S. Huie-White, E. Reiss, L. H. Harrison, L. T. Sanza, B. A. Arthington-Skaggs, and S. K. Fridkin.** 2006. Epidemiology of community-onset candidemia in Connecticut and Maryland. *Clin. Infect. Dis.* **43:**32–39.
272. **Spellberg, B. J., S. G. Filler, and J. E. Edwards, Jr.** 2006. Current treatment strategies for disseminated candidiasis. *Clin. Infect. Dis.* **42:**244–251.
273. **Strausbaugh, L. J., D. L. Sewell, T. T. Ward, M. A. Pfaller, T. Heitzman, and R. Tjoelker.** 1994. High frequency of yeast carriage on hands of hospital personnel. *J. Clin. Microbiol.* **32:**2299–2300.
274. **Sugita, T., K. Takeo, M. Ohkusu, E. Virtudazo, M. Takashima, E. Asako, F. Oshima, S. Horada, C. Yanaka, A. Nishikawa, L. Majoros, and M. Sipiczki.** 2004. Fluconazole-resistant pathogens *Candida inconspicua* and *C. norvegensis*: DNA sequence diversity of the rRNA intergenic spacer region, antifungal drug susceptibility, and extracellular enzyme production. *Microbiol. Immunol.* **48:**761–766.
275. **Sullivan, D., and D. Coleman.** 1997. *Candida dubliniensis*: an emerging opportunistic pathogen. *Curr. Top. Med. Mycol.* **8:**15–25.
276. **Swinne, D., M. Watelle, C. Suetens, K. Mertens, P. A. Fonteyne, and N. Nolard.** 2004. One-year survey of candidemia in Belgium in 2002. *Epidemiol. Infect.* **132:** 1175–1180.
277. **Tan, T. Y., A. L. Tan, N. W. S. Tee, and L. S. Y. Ng.** 2008. A retrospective analysis of antifungal susceptibilities of *Candida* bloodstream isolates from Singapore hospitals. *Ann. Acad. Med. Singapore* **37:**835–840.
278. **Taur, Y., N. Cohen, S. Dubnow, A. Paskovaty, and S. K. Seo.** 2010. The effect of antifungal timing on mortality in cancer patients with candidemia. *Antimicrob. Agents Chemother.* **54:**184–190.

279. **Tavanti, A., A. D. Davidson, N. A. Gow, M. C. Maiden, and F. C. Odds.** 2005. *Candida orthopsilosis* and *Candida metapsilosis* spp. nov. to replace *Candida parapsilosis* groups II and III. *J. Clin. Microbiol.* **43:**284–292.
280. **Tietz, H. J., V. Czaika, and W. Sterry.** 1999. Case report: osteomyelitis caused by high resistant *Candida guilliermondii*. *Mycoses* **42:**577–580.
281. **Tortorano, A. M., E. Biraghi, A. Astofi, C. Ossi, M. Tejaha, C. Farina, S. Perin, C. Bonaccorso, C. Cavanna, A. Rabalo, A. Grossi, and the FIMUA Candidemia Study Group.** 2002. European Confederation of Medical Mycology (ECMM) prospective survey of candidaemia: report from one Italian region. *J. Hosp. Infect.* **51:**297–304.
282. **Tortorano, A. M., J. Peman, H. Bernhardt, L. Klingspor, C. C. Kibbler, O. Faure, E. Biraghi, E. Canton, K. Zimmermann, S. Seaton, R. Grillot, and the ECMM Working Group on Candidaemia.** 2004. Epidemiology of candidemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study. *Eur. J. Clin. Microbiol. Infect. Dis.* **23:**317–322.
283. **Trick, W. E., S. K. Fridkin, J. R. Edwards, R. A. Hajjeh, R. P. Gaynes, and the National Nosocomial Infections Surveillance System Hospitals.** 2002. Secular trend of hospital-acquired candidemia among intensive care unit patients in the United States during 1989–1999. *Clin. Infect. Dis.* **35:**622–630.
284. **Trofa, D., A. Gascer, and J. D. Nosanchuk.** 2008. *Candida parapsilosis*, an emerging fungal pathogen. *Clin. Microbiol. Rev.* **21:**606–625.
285. **Umamaheswari, K., and T. Menon.** 2008. *Candida fermentati* from HIV patients in Chennai, South India. *Int. J. Infect Dis.* **12:**e153–e154.
286. **Utley, J. R., J. Mills, J. C. Hutchinson, L. H. Edmunds, Jr., R. G. Sanderson, and B. B. Roe.** 1973. Valve replacement for bacterial and fungal endocarditis. A comparative study. *Circulation* **48**(1 Suppl.)**:**11142–11147.
287. **Van Asbeck, E. C., Y. C. Huang, A. N. Markham, K. V. Clemons, and D. A. Stevens.** 2007. *Candida parapsilosis* fungemia in neonates: genotyping results suggest healthcare workers hands as source, and review of published studies. *Mycopathologia* **164:**287–293.
288. **Vazquez, J. A., T. Lundstrom, L. Dembry, P. Chandrasekar, D. Boikov, M. B. Parri, and M. J. Zervos.** 1995. Invasive *Candida guilliermondii* infection: in vitro susceptibility studies and molecular analysis. *Bone Marrow Transplant.* **16:**849–853.
289. **Viudes, A., J.Peman, E. Canton, P. Ubedo, J. L. Lopez-Ribot, and M. Gobernado.** 2002. Candidemia at a tertiary-care hospital: epidemiology, treatment, clinical outcome and risk factors for death. *Eur. J. Clin. Microbiol. Infect. Dis.* **21:**767–774.
290. **Wahyuningsih, R., I. M. Sah Bandar, B. Theelen, F. Hagen, G. Poot, J. F. Meis, A. Rozalyani, R. Sjam, D. Widodo, S. Djauvzi, and T. Boekhout.** 2008. *Candida nivariensis* isolated from an Indonesian human immunodeficiency virus-infected patient suffering from oropharyngeal candidiasis. *J. Clin. Microbiol.* **46:**388–391.
291. **Walsh, T. J., and W. G. Merz.** 1986. Pathologic features in the human alimentary tract associated with invasiveness of *Candida tropicalis*. *Am. J. Clin. Pathol.* **85:**498–502.
292. **Wanger, A., K. Mills, P. W. Nelson, and J. H. Rex.** 1995. Comparison of Etest and National Committee for Clinical Laboratory Standards broth microdilution method for antifungal susceptibility testing: enhanced ability to detect amphotericin B-resistant *Candida* isolates. *Antimicrob. Agents Chemother.* **39:**2520–2522.
293. **Wenzel, R. P., and C. Gennings.** 2005. Bloodstream infection due to *Candida* species in the intensive care unit: identifying especially high-risk patients to determine prevention strategies. *Clin. Infect. Dis.* **41**(Suppl. 6)**:** 5389–5393.
294. **Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel.** 1988. Hospital-acquired candidemia: the attributable mortality and excess length of stay. *Arch. Intern. Med.* **148:**2642–2645.
295. **Wey, S. B., M. Mori, M. A. Pfaller, R. F. Woolson, and R. P. Wenzel.** 1989. Risk factors for hospital-acquired candidemia. *Arch. Intern. Med.* **149:**2349–2353.
296. **Wilson, A. G., S. T. Micek, and D. J. Ritchie.** 2005. A retrospective evaluation of fluconazole for the treatment of *Candida glabrata* fungemia. *Clin. Ther.* **27:** 1228–1237.
297. **Wilson, L. S., C. M. Reyes, M. Stolpman, J. Speckman, K. Allen, and J. Beney.** 2002. The direct cost and incidence of systemic fungal infections. *Value Health* **5:**26–34.
298. **Wingard, J. R.** 1995. Importance of *Candida* species other than *C. albicans* as pathogens in oncology patients. *Clin. Infect. Dis.* **20:**115–125.
299. **Wingard, J. R., W. G. Merz, M. G. Rinaldi, T. R. Johnson, J. E. Karp, and R. Saral.** 1991. Increase in *Candida krusei* infection among patients with bone marrow transplantation and neutropenia treated prophylactically with fluconazole. *N. Engl. J. Med.* **325:**1274–1277.
300. **Wisplinghoff, H., T. Bischoff, S. M. Tallent, H. Seifert, R. P. Wenzel, and M. B. Edmond.** 2004. Nosocomial bloodstream infections in U.S. hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* **39:**309–317.
301. **Xess, I., N. Jain, F. Hasan, P. Mondal, and U. Banerjii.** 2007. Epidemiology of candidemia in a tertiary care centre of North India: 5-year study. *Infection* **35:** 256–259.
302. **Yamamura, D. L. R., C. Rotstein, L. E. Nicolle, S. Ioannou, and the Fungal Disease Registry of the Canadian Infectious Disease Society.** 1999. Candidemia at selected Canadian sites: results from the Fungal Disease Registry, 1992–1994. *CMAJ* **160:**493–499.
303. **Yang, Y. L., Y. A. Ho, H. H. Chen, M. Ho, and H. J. Lo.** 2004. Susceptibilities of *Candida* species to amphotericin B and fluconazole: the emergence of fluconazole resistance in *Candida tropicalis*. *Infect. Control Hosp. Epidemiol.* **25:**60–64.
304. **Yang, Y. L., S. Y. Li, H. H. Chang and H. J. Lo.** 2005. Susceptibilities to amphotericin B and fluconazole of *Candida* species in TSARY 2002. *Diagn. Microbiol. Infect. Dis.* **51:**179–183.
305. **Yang, Y. L., H. H. Chang, H. J. Lo, and the TSARY Hospitals.** 2006. Distribution and antifungal susceptibility of *Candida* species isolated from different age populations in Taiwan. *Med. Mycol.* **44:**237–242.
306. **Yang, Y. L., A. H. Wang, C. W. Wang, W. T. Cheng, S. Y. Li, H. J. Lo, and the TSARY Hospitals.** 2008. Susceptibilities to amphotericin B and fluconazole of *Candida* species in Taiwan Surveillance of Antimicrobial Resistance of Yeasts 2006. *Diagn. Microbiol. Infect. Dis.* **61:** 175–180.
307. **Yoo, J. I., C. W. Choi, K. M. Lee, Y. K. Kim, T. U. Kim, E. C. Kim, S. I. Joo, S. H. Yun, Y. S. Lee, and B. S. Kim.** 2009. National surveillance of antifungal susceptibility of *Candida* species in South Korean hospitals. *Med. Mycol.* **47:**554–558.
308. **Yoon, S. A., J. A. Vazquez, P. E. Stefan, J. D. Sobel, and R. A. Akins.** 1999. High-frequency in vitro reversible

switching of *Candida lusitaniae* clinical isolates from amphotericin B susceptibility to resistance. *Antimicrob. Agents Chemother.* **43:**836–845.

309. **Zaoutis, T. E., J. Argon, J. Chu, J. A. Berlin, T. J. Walsh, and C. Feudtner.** 2005. The epidemiology and attributable outcomes of candidemia in adults and children hospitalized in the United States: a propensity analysis. *Clin. Infect. Dis.* **41:**1232–1239.
310. **Zilberberg, M. D., A. F. Shorr, and M. Kollef.** 2008. Secular trends in candidemia-related hospitalization in the United States, 2000–2005. *Infect. Control Hosp. Epidemiol.* **29:**978–980.
311. **Zilberberg, M. D., A. F. Shorr, and M. H. Kollef.** 2008. Growth and geographic variation in hospitalizations with resistant infections, United States, 2000–2005. *Emerg. Infect. Dis.* **14:**1756–1758.

COOL TOOLS FOR RESEARCH

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch30

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Cool Tools 1: Development and Application of a *Candida albicans* Two-Hybrid System

BRAM STYNEN, PATRICK VAN DIJCK, AND HÉLÈNE TOURNU

THE YEAST TWO-HYBRID SYSTEM

Interactions between proteins lie at the core of many crucial processes in the cell, including signal transduction, translation, transcription, DNA replication, and metabolic pathways. Many methods have been developed to investigate protein-protein interactions, which can be clustered in biophysical (e.g., surface plasmon resonance [22]), biochemical (e.g., tandem affinity purification [18]), computational (e.g., protein-protein docking [1]), and genetic (e.g., bimolecular fluorescence complementation [12]) approaches. As a genetic approach, the yeast two-hybrid system has been highly successful in confirming and discovering protein-protein interactions (7). The principle of this method is based on the creation of two hybrid proteins, one fused to a DNA-binding domain (DBD; the "bait") and the other one fused to a transcription activation domain (AD; the "prey"). When the two proteins bind to each other, a functional transcription factor is assembled that allows for the expression of one or more reporter genes, each with a promoter region that is recognized by the bait DBD. Since its development in 1989, the yeast two-hybrid system has gone through many improvements and alternative approaches. Several chromogenic, prototrophic, fluorescent, and antibiotic resistance reporter genes are available (4, 6, 8, 11, 13, 23, 26). The most popular DBDs are the N-terminal Gal4 DBD and the *Escherichia coli* LexA repressor, which, respectively, bind Gal4 upstream activating sequences and LexA operator sequences in the promoter region of the reporter genes (7, 8). The most common options for the AD in the prey are, with increasing sensitivity, the bacterial B42 AD (8), the *Saccharomyces cerevisiae* Gal4 AD (7), and the viral VP16 protein (26). As the expression of the bait and prey fusion genes greatly influences sensitivity and specificity (14), transcription of both genes is controlled by strong (full-length *ADH1* promoter), weak (truncated *ADH1* promoter), or inducible (*GAL1* promoter) promoters, on a high-copy-number (2μm-based) or low-copy-number (CEN-based) plasmid (10). Optionally, bait and prey genes include epitope tags (e.g., V5 and hemagglutinin [HA] [8, 28]) for expression analysis and nuclear localization sequences (NLSs) (simian virus 40 [SV40] NLS) for proper translocation to the nucleus (21).

To overcome the limitations of *S. cerevisiae* as a host organism, two-hybrid systems were created in other eukaryotes such as *Arabidopsis thaliana*, *Drosophila melanogaster*, and *Caenorhabditis elegans* and in mammalian cells (9, 15, 16, 27). These natural contexts ensure correct intron splicing and posttranslational modifications, not always available in the model yeast. For organisms with an alternative codon usage, such as the human fungal pathogen *Candida albicans*, protein-protein interaction analysis in *S. cerevisiae* is not optimal due to possible erroneous translation of the proteins and subsequent failure to detect interactions. Recently, an alternative *C. albicans* two-hybrid system was developed, called the vesicle capture assay (2). This method is based upon the construction of two hybrid proteins, one protein that is fused to the vesicle targeted protein Vps32 and one protein fused to green fluorescent protein (GFP). Localization of GFP to endocytic vesicle surfaces is used as a readout for interaction analysis. We describe here a new *C. albicans* two-hybrid system based upon the classic approach of transcription factor complementation (24). An overview of the *C. albicans* two-hybrid system is provided in Fig. 1.

DEVELOPMENT AND APPLICATION OF A *CANDIDA ALBICANS* TWO-HYBRID SYSTEM

To develop a two-hybrid system suitable for *Candida albicans*, all components of a yeast two-hybrid system needed to be included, with the requirement that each component was functional in *C. albicans*. Russell and Brown (20) described the use in a one-hybrid system of *Staphylococcus aureus* LexA in *C. albicans*, as a DBD which binds to a LexA operator sequence placed upstream of the reporter gene *Streptococcus thermophilus lacZ* (25). As a result, the ability

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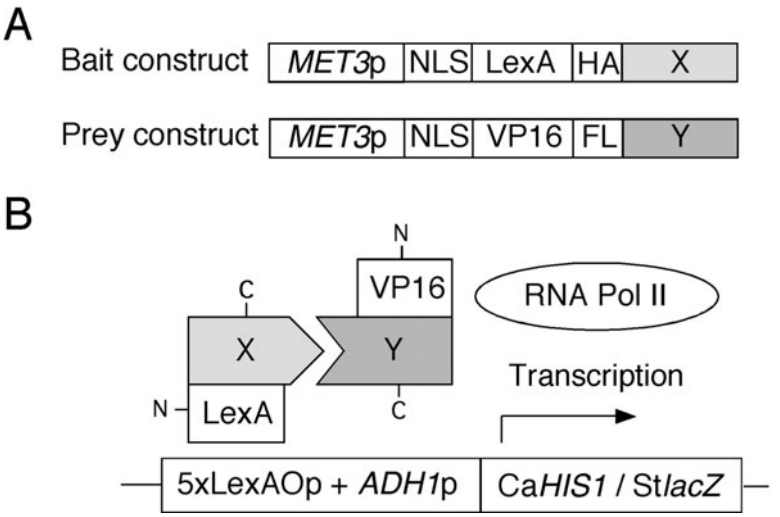

FIGURE 1 Overview of the C. *albicans* two-hybrid system. (A) Bait and prey constructs. Bait and prey genes are expressed under the control of an inducible *MET3* promoter. The bait fusion protein includes *S. aureus* LexA as DNA binding domain, and the prey protein comprises a codon-optimized VP16 activation domain. Both constructs contain SV40 NLSs and epitope tags (HA tag for bait and FLAG [FL] tag for prey). (B) Interaction leading to reporter gene expression. The bait protein is located at the promoter region of reporter genes *S. thermophilus lacZ* and C. *albicans HIS1* by the binding of *S. aureus* LexA with its target LexA operator (LexAOp) sequences. When proteins X and Y interact, the VP16 activation domain is brought in close proximity to the reporter genes, resulting in their transcription. Analysis of an interaction is performed on selective medium (*HIS1*) or by a galactosidase assay (*lacZ*). 10.1128/9781555817176.ch30f1

of the protein of interest, bound to the LexA DBD, to activate transcription can be evaluated by a colorimetric assay. For the two-hybrid strain (SC2H3), *S. thermophilus lacZ* and a second reporter gene, C. *albicans HIS1*, were introduced in strain SN152 (17) for double confirmation of an interaction (Fig. 2A). For the activation domain, several candidates, including three C. *albicans* transcription factors (Gcn4, Tac1, and Ino2) and two traditional yeast two-hybrid activation domains (B42 and VP16), were tested, and a codon-optimized version of VP16 proved to be the most sensitive AD. Both bait and prey genes were placed under the control of the inducible *MET3* promoter (3). Epitope tags (an HA tag for the bait and a FLAG tag for the prey) were included right upstream of the multiple cloning site (Fig. 1). Finally, at the N-terminal end, the SV40 NLS was added to complete the constructs. Both bait (pC2HB) and prey (pC2HP) plasmids can be integrated in a defined genomic region by a restriction digest with NotI, which rarely cuts the genomic DNA of C. *albicans* (a NotI sequence site is present in only four open reading frames). Finally, the sensitivity of the system was improved by the addition of five LexA operator sequences in the promoter region of the reporter genes (plasmid maps are shown in Fig. 2B).

As an example, interaction was demonstrated between the proteins Kis1 and Snf4 (24), both components of the Snf1 kinase complex, which regulates carbohydrate metabolism (5). In the assay, Kis1 interacted with Snf4 in both directions, as seen by a spot assay on synthetic complete medium without histidine and by a galactosidase assay (Fig. 3A). Taking advantage of the epitope tags fused to the bait and prey proteins, coimmunoprecipitation experiments confirmed that Kis1 indeed interacts with Snf4 (Fig. 3B).

In conclusion, this C. *albicans* two-hybrid system can be used to enhance our knowledge of protein-protein interactions in C. *albicans*. Although only one-to-one interactions were tested until now, the method should be compatible for screening experiments due to the selective step involved.

GENERAL PROTOCOL FOR AN INTERACTION ASSAY

1. Clone genes of interest in the bait and prey plasmids, which both contain a kanamycin resistance marker for positive selection in *E. coli*. StuI and AscI restriction sites (Fig. 2B) are recommended for cloning, as they are present in the multiple cloning site of both vectors and the majority of C. *albicans* genes do not contain these sites.

2. Transform strain SC2H3 with NotI-digested bait plasmids pC2HB (empty plasmid) and pC2HB with the gene of interest included. Selection for integration is enabled by the marker gene *Candida maltosa LEU2*. Correct integration of the bait plasmid can be verified by PCR with primers 5′-ATGAAAGGACAATCACGAAGCC (within the genomic DNA) and 5′-CGGAGAACCTGCGTGCAATCC (within the kanamycin marker gene), resulting in a 2,200-bp fragment. Integration in the correct location is very efficient for both the bait and prey plasmid (>90%).

3. Transform the resulting bait strains with NotI-digested prey plasmids pC2HP (empty plasmid) and pC2HP with the gene of interest included. Selection for integration is enabled by the marker gene *Candida dubliniensis ARG4*. Use for each construct two bait transformants. Correct integration of the bait and prey plasmids can be verified by PCR with the primers 5′-AATACACGACCTAATATTGG (within the genomic DNA) and 5′-GACTTATGTCTAATACCTCC (within marker gene Cd*ARG4*), resulting in a 1,200-bp fragment.

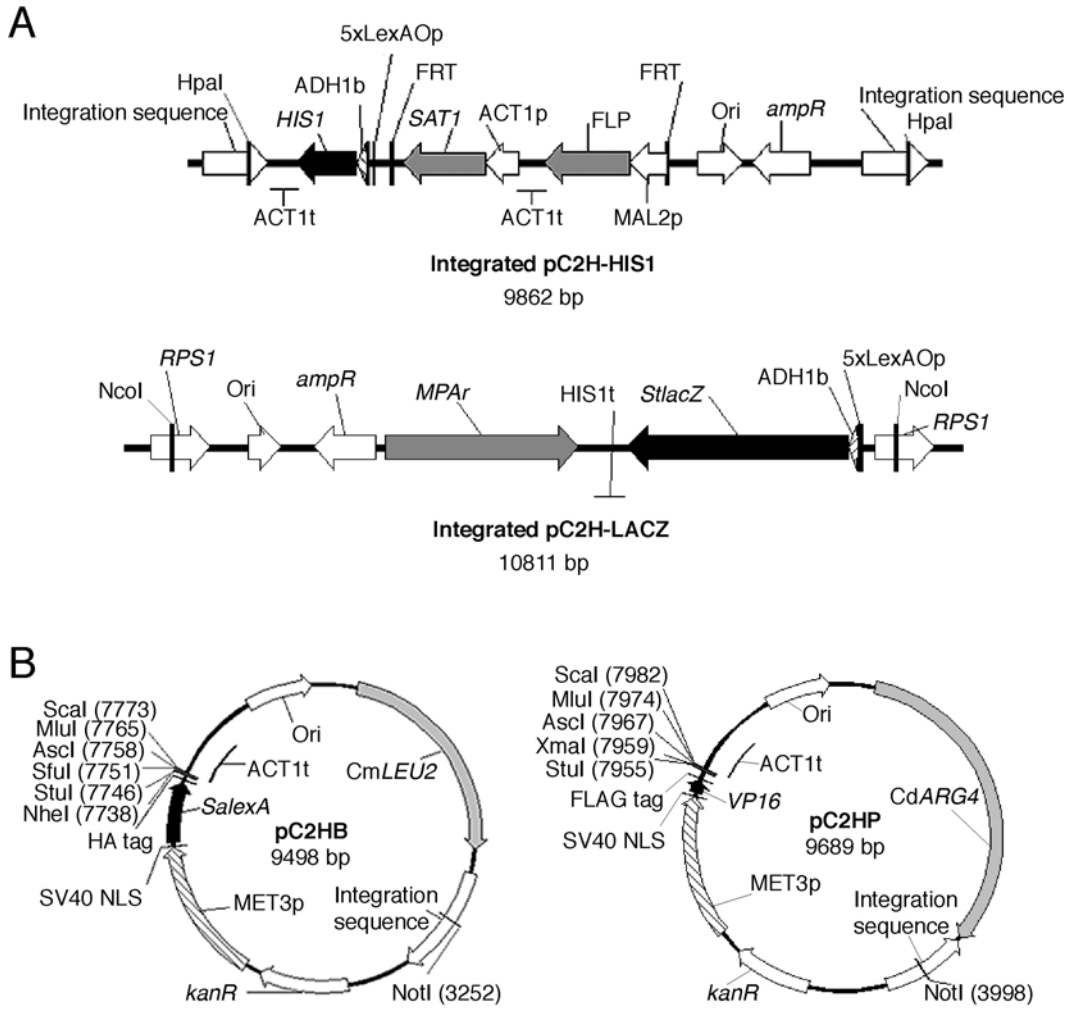

FIGURE 2 (A) The two-hybrid strain SC2H3 contains two integrated reporter constructs. Plasmid pC2H-HIS1 includes reporter gene *HIS1* in front of a basal *ADH1* promoter and five LexA operator (LexAOp) sequences for binding of the bait protein. Plasmid pC2H-LACZ contains reporter gene *lacZ* preceded by a basal *ADH1* promoter sequence and five LexAOp sequences for binding of the bait protein. The complete plasmid is flanked by restriction sites used for integration. Linearized pC2H-HIS1 integrates at the HpaI site located between genes *PGA59* and *PGA62* on chromosome 4, while pC2H-LACZ integrates at the *RPS1* locus on chromosome 1, at the NcoI site. ADH1b, basal promoter of *ADH1*; *ampR*, ampicillin resistance gene; Ori, origin of replication; t, terminator; p, promoter. (B) Bait and prey plasmids, pC2HB and pC2HP. A bait gene of interest (left panel) is cloned into the multiple cloning site downstream of the DNA-binding protein S*alexA* and under the control of the *MET3* promoter. The pC2HB plasmid is integrated between loci *XOG1* and *HOL1* on chromosome 1 after linearization at restriction site NotI, and selection is obtained with the auxotrophic marker *LEU2* from *Candida maltosa*. For the prey plasmid pC2HP (right panel), a prey gene of interest is cloned into the multiple cloning site, downstream of AD VP16. The *MET3* promoter controls expression of the prey gene. An integration sequence, situated in the intergenic region between genes *RXT3* and *ORF19.3569* on chromosome 2, makes insertion in the genome possible after linearization of the plasmid at restriction site NotI. C. *dubliniensis ARG4* is the auxotrophic marker for transformation. Reprinted from *Nucleic Acids Research* (24) with permission of the publisher. 10.1128/9781555817176.ch30f2

4. Streak transformants or perform a spot assay on selective medium (synthetic complete without histidine). To modulate the expression of the bait and prey genes, media with small amounts of methionine (0.15 mM) for weak expression or without methionine for fully induced expression can be used. Include all the negative controls. In the case of a positive result, clear growth on selective medium can be observed after 1 and up to 7 days. A positive interaction can be expected when a minimum of 30% of transformants grow on selective medium.

5. Grow your strains overnight in synthetic complete (SC) medium, reinoculate the washed cells in SC medium without methionine at an optical density at 595 nm of 1 in 5 ml, incubate for 6 to 7 h at 30°C, and collect the cells.

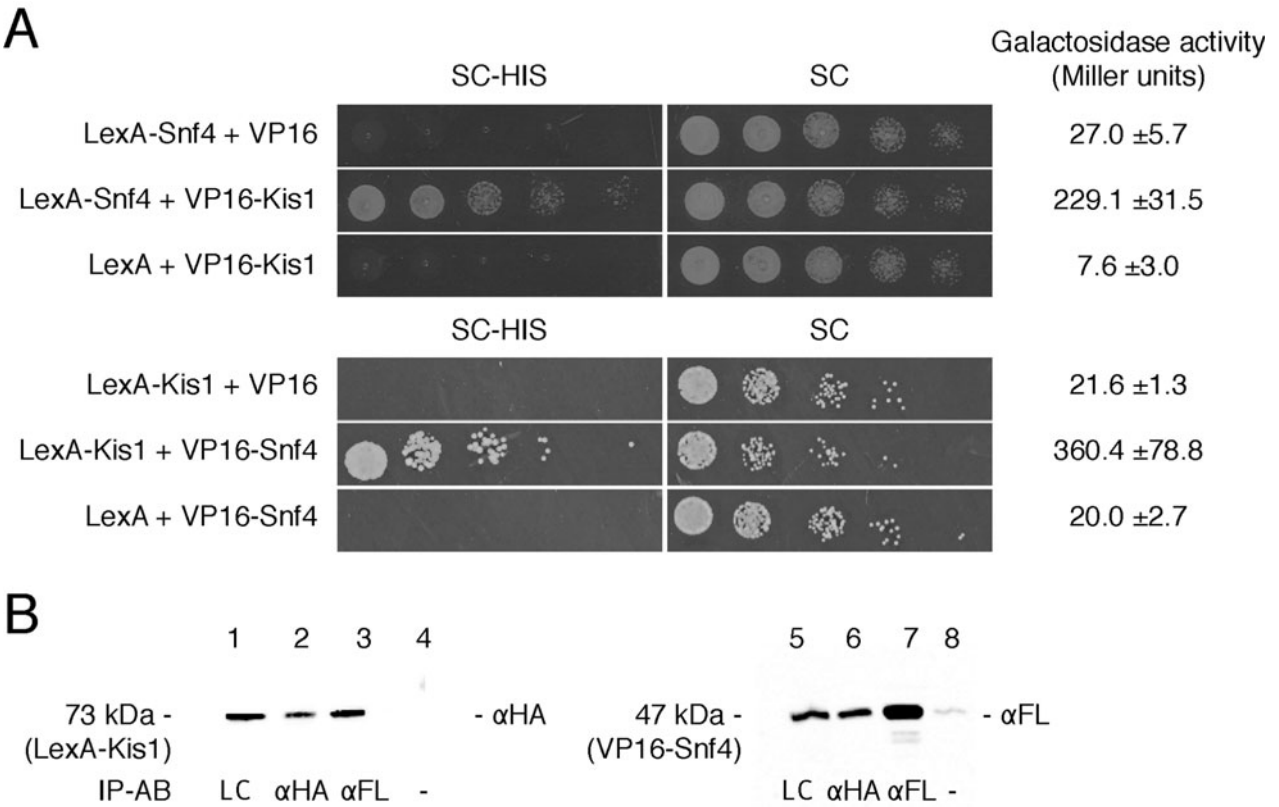

FIGURE 3 (A) Two-hybrid interaction of *C. albicans* Kis1 with Snf4. Kis1 and Snf4 interact with each other as shown by growth of SC2H3 on SC-HIS and a high galactosidase activity. For the *HIS1* reporter assay, cells were incubated for up to 2 days. (*B*) Coimmunoprecipitation of Kis1 and Snf4. The interaction between bait LexA-HA-Kis1 (73 kDa) and prey VP16-FLAG-Snf4 (47 kDa) is confirmed in a coimmunoprecipitation experiment. Total protein concentrations were equal for each sample. Lane 1, loading control of Kis1; lane 2, immunoprecipitation of Kis1 with anti-HA antibodies; lane 3, coimmunoprecipitation of Kis1 with anti-FLAG antibodies; lane 4, negative control of Kis1 immunoprecipitation without antibodies; lane 5, loading control of Snf4; lane 6, coimmunoprecipitation of Snf4 with anti-HA antibodies; lane 7, immunoprecipitation of Snf4 with anti-FLAG antibodies; lane 8, negative control of Snf4 immunoprecipitation without antibodies. The antibodies used for Western blotting are indicated on the right side of each blot, and the antibodies for immunoprecipitation are shown below the blot. IP-AB, antibody used for immunoprecipitation; LC, loading control; αFL, anti-FLAG antibody; αHA, anti-HA antibody. Reprinted from *Nucleic Acids Research* (24) with permission of the publisher. 10.1128/9781555817176.ch30f3

Perform a β-galactosidase assay as described previously (19), at 37°C.

REFERENCES

1. **Aloy, P., and R. B. Russell.** 2006. Structural systems biology: modeling protein interactions. *Nat. Rev. Mol. Cell Biol.* **7:**188–197.
2. **Boysen, J. H., S. Fanning, J. Newberg, R. F. Murphy, and A. P. Mitchell.** 2009. Detection of protein-protein interactions through vesicle targeting. *Genetics* **182:**33–39.
3. **Care, R. S., J. Trevethick, K. M. Binley, and P. E. Sudbery.** 1999. The *MET3* promoter: a new tool for *Candida albicans* molecular genetics. *Mol. Microbiol.* **34:**792–798.
4. **Cormack, R. S., K. Hahlbrock, and I. Somssich.** 1998. Isolation of putative plant transcriptional coactivators using a modified two-hybrid system incorporating a GFP reporter gene. *Plant J.* **14:**685–692.
5. **Corvey, C., P. Koetter, T. Beckhaus, J. Hack, S. Hofmann, M. Hampel, T. Stein, M. Karas, and K. D. Entian.** 2005. Carbon source-dependent assembly of the Snf1p kinase complex in *Candida albicans*. *J. Biol. Chem.* **280:**25323–25330.
6. **Endo, M., K. Takesako, I. Kato, and H. Yamaguchi.** 1997. Fungicidal action of aureobasidin A, a cyclic depsipeptide antifungal antibiotic. *Antimicrob. Agents Chemother.* **41:**672–676.
7. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. *Nature* **340:**245–246.
8. **Gyuris, J., E. Golemis, H. Chertkov, and R. Brent.** 1993. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75:**791–803.
9. **Hiatt, S. M., Y. J. Shyu, H. M. Duren, and C. D. Hu.** 2008. Bimolecular fluorescence complementation (BiFC) analysis of protein interactions in *Caenorhabditis elegans*. *Methods* **45:**185–191.
10. **James, P.** 2001. Yeast two-hybrid vectors and strains. *Methods Mol. Biol.* **177:**41–84.
11. **James, P., J. Halladay, and E. A. Craig.** 1996. Genomic libraries and a host strain designed for highly efficient two-hybrid selection in yeast. *Genetics* **144:**1425–1436.

12. **Kerppola, T. K.** 2006. Design and implementation of bimolecular fluorescence complementation (BiFC) assays for the visualization of protein interactions in living cells. *Nat. Protoc.* **1:**1278–1286.
13. **Le Douarin, B., B. Pierrat, E. vom Baur, P. Chambon, and R. Losson.** 1995. A new version of the two-hybrid assay for detection of protein-protein interactions. *Nucleic Acids Res.* **23:**876–878.
14. **Legrain, P., M. C. Dokhelar, and C. Transy.** 1994. Detection of protein-protein interactions using different vectors in the two-hybrid system. *Nucleic Acids Res.* **22:** 3241–3242.
15. **Luo, Y., A. Batalao, H. Zhou, and L. Zhu.** 1997. Mammalian two-hybrid system: a complementary approach to the yeast two-hybrid system. *BioTechniques* **22:**350–352.
16. **Mon, H., R. Sugahara, S. M. Hong, J. M. Lee, Y. Kamachi, Y. Kawaguchi, and T. Kusakabe.** 2009. Analysis of protein interactions with two-hybrid system in cultured insect cells. *Anal. Biochem.* **392:**180–182.
17. **Noble, S. M., and A. D. Johnson.** 2005. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. *Eukaryot. Cell* **4:**298–309.
18. **Puig, O., F. Caspary, G. Rigaut, B. Rutz, and E. Bouveret.** 2001. The tandem affinity purification (TAP) method: a general procedure of protein complex purification. *Methods* **24:**218–229.
19. **Rose, M., and D. Botstein.** 1983. Construction and use of gene fusions to LacZ (β-galactosidase) which are expressed in yeast. *Methods Enzymol.* **101:**167–180.
20. **Russell, C. L., and A. J. P. Brown.** 2005. Expression of one-hybrid fusions with *Staphylococcus aureus* lexA in *Candida albicans* confirms that Nrg1 is a transcriptional repressor and that Gcn4 is a transcriptional activator. *Fungal Genet. Biol.* **42:**676–683.
21. **Schneider, J., C. Schindewolf, K. van Zee, and E. Fanning.** 1988. A mutant SV40 large T antigen interferes with nuclear localization of a heterologous protein. *Cell* **54:** 117–125.
22. **Schuck, P.** 1997. Reliable determination of binding affinity and kinetics using surface plasmon resonance biosensors. *Curr. Opin. Biotechnol.* **8:**498–502.
23. **Serebriiskii, I., V. Khazak, and E. A. Golemis.** 1999. A two-hybrid dual bait system to discriminate specificity of protein interactions. *J. Biol. Chem.* **274:**17080–17087.
24. **Stynen, B., P. Van Dijck, and H. Tournu.** 2010. A CUG codon adapted two-hybrid system for the pathogenic fungus *Candida albicans*. *Nucleic Acids Res.* **38:**e184.
25. **Uhl, M. A., and A. D. Johnson.** 2001. Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*. *Microbiology* **147:**1189–1195.
26. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74:**205–214.
27. **Walter, M., C. Chaban, K. Schutze, O. Batistic, K. Weckermann, C. Nake, D. Blazevic, C. Grefen, K. Schumacher, C. Oecking, K. Harter, and J. Kukla.** 2004. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* **40:**428–438.
28. **Yavuzer, U., and C. R. Goding.** 1995. pWITCH: a versatile two-hybrid assay vector for the production of epitope/activation domain-tagged proteins both *in vitro* and in yeast. *Gene* **165:**93–96.

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Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch31

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Cool Tools 2: Development of a *Candida albicans* Cell Surface Protein Microarray

A. BRIAN MOCHON

The cell surface of *Candida albicans* is necessary for colonization of the human host and is also the target of the immune system when *C. albicans* enters the bloodstream as an opportunistic pathogen. However, relatively little is known regarding the molecular and cellular dynamics of *C. albicans* during commensal colonization and the development of pathogenesis. Thus, information on in vivo gene expression would provide insight into how *C. albicans* interacts with host cells during the transition from commensal colonization to an opportunistic pathogen in the immunocompromised host. Nonetheless, in vivo transcription profiling of *C. albicans* during commensal colonization or candidemia is technically challenging (6). Thus, profiling of antibody responses during colonization and infection offers an alternative approach that can overcome technical challenges of in vivo transcription profiling.

To investigate the establishment of humoral immunity during commensal sensitization, as well as the adaptive immune response to candidemia, my laboratory developed a *C. albicans* cell surface protein microarray (19). In that study, my group observed strong immunoglobulin G (IgG) responses to many proteins known to be induced and/or required for *C. albicans* invasion of epithelial and endothelial cells in both candidemia patients and noncandidemia controls, including healthy individuals. These findings indicate that the immunocompetent host exists in permanent host-pathogen interplay with commensal *C. albicans* (Table 1). The study also identified cell surface antigens that are specific to different phases (i.e., acute phase and early and mid-convalescence) of candidemia. The study identified a set of 13 cell surface antigens capable of distinguishing acute candidemia from healthy individuals and uninfected hospital patients with commensal colonization (Table 2). Interestingly, a large proportion of these cell surface antigens are involved in either oxidative stress or drug resistance. In addition, the assay identified 33 antigenic proteins that are enriched in convalescent-phase sera of candidemia patients (Table 3). Intriguingly, within this subset was found an increase in antigens associated with heme-associated iron acquisition. These findings have important implications for the mechanisms of *C. albicans* colonization and the development of systemic infection.

The development of the antigenic profiles over the course of candidiasis (acute infection, early convalescence, and mid-convalescence) may also provide insight into a protective humoral response against *C. albicans*. Even though previous sensitization to commensal colonization does not limit mortality or even morbidity in patients, experimental studies have identified protective antibodies against hematogenously disseminated candidiasis, such as heat shock protein 90 (Hsp90) or β-mannan (12, 13, 18, 21). Studies will need to address whether the serodiagnostic antigens identified in this study could provide protection from hematogenously disseminated candidiasis and whether serological differences exist between superficial (i.e., thrush and vaginal candidiasis) and systemic infections (19). Finally, the increasing number of candidal infections necessitates the assay development of protein microarrays that include the whole proteome of not just *C. albicans* but also other pathogenic *Candida* species, as these studies may elucidate additional serodiagnostic antigens and/or vaccine candidates.

CONSTRUCTION OF *CANDIDA ALBICANS* PROTEIN MICROARRAY

Collection of Candidemia and Control Sera

Candidemia was defined as the recovery of *C. albicans* from blood cultures. Sera from candidemia patients were collected as previously published (7). Briefly, sera from candidemia patients were obtained from the earliest possible date on or after the day that the first positive cultures were obtained. The median time from the date of positive culture to serum collection was 2 days. A subset of the candidemia patients were monitored through acute infection (days 0 to 14) to early convalescent (week 4) and mid-convalescent (week 12) infection. Included in the study were sera from 12 uninfected hospital patients and 50 healthy individuals who had no evidence of candidiasis as the negative control

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TABLE 1 The 40 most serodominant antigens in acute candidemia patients[a]

Name		Description	Mean antigen reactivity (+/– SEM)		BH-adjusted *P* value
Systematic	Common		Candidemia patients	Negative controls	
19.4257 (1)	Int1 (1)	Integrin-like protein	35,380 (11,412–109,691)	32,163 (12,268–84,323)	0.853
19.4421	Cwh41	Glucosidase	32,758 (13,701–78,323)	40,288 (16,253–99,864)	0.642
19.6420	Pga13	Unknown function	27,057 (10,402–70,383)	40,478 (25,960-63,115)	0.116
19.5636	Rbt5	Hemoglobin utilization	19,351 (4,882–76,695)	12,787 (3,956–41,338)	0.419
19.1321	Hwp1	Hyphal wall protein	19,136 (4,604–79,530)	15,974 (4,407–57,899)	0.776
19.6763	Slk19	Unknown function	18,546 (6,904–49,823)	15,590 (4,535–53,590)	0.769
19.6481	Yps7	Aspartic-type peptidase	14,170 (4,202–47,787)	15,103 (4,189–54,451)	0.915
19.1816 (2)	Als3 (2)	Agglutinin-like protein; iron assimilation	13,292 (5,047–35,009)	16,960 (7,384–38,953)	0.531
19.7298 (1)	Chs2 (1)	Chitin synthase	12,221 (5,271–28,335)	20,418 (13,475–30,940)	0.0361
19.5788 (1)	Eft2 (1)	Elongation factor 2	11,973 (3,530–40,612)	9,139 (3,878–21,538)	0.504
19.3988	Ipf9655	Unknown function	11,642 (5,339–25,386)	12,904 (6,096–27,318)	0.806
19.7565	Gnp3	Glutamine permease	8,919 (2,049–38,826)	6,306 (1,686–23,592)	0.535
19.5632	Phr3	Glucanosyltransferase	8,845 (2,542–30,768)	12,600 (4,498–35,298)	0.436
19.3374	Ece1	Unknown function	8,571 (1,967–37,354)	3,401 (702–16,481)	0.105
19.4565	Bgl2	Glucanosyltransferase	8,437 (4,642–15,334)	5,564 (3,022–10,244)	0.116
19.886	Pan1	Actin cytoskeleton-regulatory complex	8,368 (2,924–23,953)	14,845 (5,032–43,796)	0.177
19.4257 (2)	Int1 (2)	Integrin-like protein	8,256 (2,986–22,828)	12,856 (4,405–37,517)	0.321
19.5095 (2)	Osh2 (2)	Oxysterol-binding protein	8,103 (2,696–24,359)	6,953 (1,832–26,385)	0.806
19.5095 (1)	Osh2 (1)	Oxysterol-binding protein	8,070 (2,178–29,899)	10,254 (3,080–34,142)	0.667
19.4784 (2)	Crp1 (2)	Copper transporter	7,937 (3,951–15,943)	7,260 (4,836–10,899	0.751
19.2787	Pry1	Unknown function	7,813 (4,172–14,634)	13,674 (9,461–19,764)	0.00451
19.5588	Pga60	Unknown function	7,477 (4,350–12,852)	8,332 (3,111–22,311)	0.808
19.1671	Utr2	Glycosidase	7,466 (4,590–12,144)	8,456 (6,440–11,102)	0.479
19.2003	Hnm1	Choline transporter	7,385 (4,798–11,367)	8,333 (6,454–10,759)	0.470
19.4975 (2)	Hyr1 (2)	Hyphal wall protein	7,221 (3,177–16,411)	4,665 (2,527–8,610)	0.0814
19.7251	Wsc4	Unknown function	6,906 (3,926–12,150)	9,603 (7,001–13,172)	0.0457
19.3174 (1)	Cdc24 (1)	GDP-GTP exchange factor	6,888 (3,544–13,387)	10,759 (7,607–15,215)	0.0106
19.575 (2)	Hyr3 (2)	Unknown function	6,846 (2,378–19,712)	5,695 (2,069–15,676)	0.688
19.932	Dnf2	Phospholipid translocase	6,766 (4,655–9,836)	7,475 (4,596–12,156)	0.688
19.5672	Mep2	Ammonium permease	6,743 (3,949–11,514)	7,885 (3,644–17,059)	0.652
19.4899 (1)	Gca1 (1)	Glucoamylase	6,640 (2,881–15,302)	9,427 (5,866–15,151)	0.116
19.3225 (1)	Cwh43 (1)	Unknown function	6,537 (4,850–8,809)	6,671 (4,821–9,231)	0.921
19.1415	Fre10	Ferric reductase	6,528 (4,408–9,667)	9,450 (6,428–13,893)	0.0214
19.5736 (2)	Als5 (2)	Agglutinin-like protein	6,231 (2,848–13,634)	7,448 (3,933–14,103)	0.556
19.5741 (2)	Als1 (2)	Agglutinin-like protein	5,795 (2,489–13,495)	10,653 (4,371–25,966)	0.0649
19.3256 (2)	Sln1 (2)	Histidine kinase; osmosensor	5,561(1,535–20,139)	2,866 (1,695–4,846)	0.00878
19.1357	Fcy21	Purine-cytosine permease	5,445 (3,827–7,748)	5,895 (4,377–7,937)	0.678
19.1690	Tos1	α-Agglutinin anchor subunit	5,412 (3,340–8,768)	3,994 (2,535–6,291)	0.116
19.4215	Fet34	Multicopper ferroxidase	5,372 (2,502–11,534)	6,232 (2,745–14,147)	0.689
19.5112	Tkl1	Transketolase	5,337 (2,756–10,335)	7,143 (3,938–12,957)	0.261

[a]Reprinted from reference 19 with permission.

groups (no clinical or microbiological evidence of candidemia). All sera were collected and stored at -70ºC.

PCR Amplification of Genes Encoding *C. albicans* Cell Surface Proteins

To search for genes encoding C. *albicans* cell surface proteins, the *Candida* Genome Database (CGD) (http://www.candidagenome.org) was mined. Cell surface proteins were selected from the CGD using keywords such as "cell surface," "plasma membrane," and "cell wall." The CGD annotation of cell surface proteins is based on published experiments (1, 5, 9, 11, 20, 27), function-based prediction of cellular localization, and sequence prediction. Known antigenic proteins were also included as controls (Bgl2, Eno1, Pgk1, Gap1, Cdc19, Tkl1, Hsp90, and members of the Hsp70 family) (22, 23). Coding regions of the genes were PCR amplified from the clinical isolate SC5314 of C. *albicans*. A high-throughput in vivo transformation system

TABLE 2 Antigenic biomarkers of acute candidemia patients[a]

Name			Mean antigen reactivity (+/– SEM)		BH-adjusted	
Systematic	Common	Description	Candidemia patients	Negative controls	*P* value	AUC
19.6000 (3)	Cdr1 (3)	Drug transporter	2,842 (1,295–6,234)	837 (440–1,593)	1.04E-07	0.873
19.1844	Cfl91	Ferric reductase	3,522 (778–15,943)	752 (329–1,719)	1.47E-06	0.792
19.5079 (3)	Cdr4 (3)	Drug transporter	4,433 (2,104–9,341)	2,089 (1,285–3,394)	1.07E-04	0.777
19.5742 (2)	Als9 (2)	Agglutinin-like protein	4,233 (2,167–8,267)	2,025 (1,037–3,956)	1.96E-03	0.786
19.3575	Cdc19	Pyruvate kinase	3,235 (1,364–7,673)	1,704 (960–3,024)	6.10E-03	0.755
19.5181 (2)	Nik1 (2)	Osmosensor	2,420 (975–6,008)	1,198 (605–2,372)	6.51E-03	0.722
19.5384 (2)	Chs8 (2)	Chitin synthase	2,306 (984–5,402)	1,267 (739–2,170)	6.69E-03	0.750
19.6595	Rta4	Phospholipid transporter	3,011 (1,802–5,030)	1,506 (690–3,285)	6.94E-03	0.764
19.3256 (2)	Sln1 (2)	Osmosensor	5,561 (1,535–20,139)	2,866 (1,695–4,846)	8.78E-03	0.630
19.600 (2)	Trk1 (2)	Potassium transporter	2,780 (1,483–5,211)	1,652 (890–3,066)	0.0214	0.784
19.1783 (3)	Yor1 (3)	Drug transporter	2,566 (1,024–6,427)	1,593 (989–2,565)	0.0269	0.651
19.6926	Csc25	Guanyl-nucleotide exchange factor	2,507 (1,710–3,675)	1,563 (834–2,930)	0.0362	0.735
19.5902	Ras2	RAS signal transduction	3,005 (1,872–4,824)	2,032 (1,234–3,348)	0.0417	0.704

[a]Reprinted from reference 19 with permission. AUC, area under the curve.

TABLE 3 Antigenic biomarkers of early-convalescent-phase and mid-convalescent-phase candidemia patients[a]

Name			Mean antigen reactivity (+/– SEM)		BH-adjusted	
Systematic	Common	Description	Candidemia patients	Negative controls	*P* value	AUC
19.1844	Cfl91	Ferric reductase	10,699 (4,436–25,802)	752 (329–1,719)	0	0.969
19.323 (2)	Drs23 (2)	Phospholipid translocase	3,249 (1,812–5,826)	707 (439–1,137)	4.21E-14	0.960
19.5079 (3)	Cdr4 (3)	Drug transporter	9,831 (5,588–17,295)	2,089 (1,285–3,394)	2.28E-13	0.957
19.2296 (2)	Ipf25023 (2)	Unknown function	3,128 (1,446–6,763)	461 (234–908)	3.46E-13	0.945
19.1783 (3)	Yor1 (3)	Drug transporter	6,719 (3,759–12,009)	1,593 (989–2,565)	6.91E-13	0.970
19.6000 (3)	Cdr1 (3)	Drug transporter	3,635 (2,139–6,178)	837 (440–1,593)	1.00E-09	0.964
19.7414 (2)	Als6 (2)	Agglutinin-like protein	2,868 (1,176–6,990)	883 (569–1,372)	4.56E-09	0.917
19.1800	Vps62	Unknown function	19,259 (10,436–35,543)	5,032 (2,139–11,841)	1.33E-05	0.896
19.5759 (3)	Snq2 (3)	Drug transporter	2,580 (1,740–3,825)	1,313 (918–1,877)	1.82E-05	0.896
19.5742 (2)	Als9 (2)	Agglutinin-like protein	5,504 (3,654–8,290)	2,025 (1,037–3,956)	3.14E-05	0.913
19.5636	Rbt5	Hemoglobin utilization	67,414 (30,441–149,296)	12,787 (3,956–41,338)	5.45E-05	0.878
19.600 (2)	Trk1 (2)	Potassium transporter	4,189 (2,131–8,233)	1,652 (890–3,066)	1.07E-04	0.922
19.4565	Bgl2	Glucanosyltransferase	13,462 (7,309–24,793)	5,564 (3,022–10,244)	2.53E-04	0.866
19.5181 (2)	Nik1 (2)	Osmosensor	3,090 (1,075–8,878)	1,198 (605–2,372)	4.58E-04	0.945
19.6595	Rta4	Phospholipid transporter	3,847 (2,330–6,354)	1,506 (690–3,285)	4.99E-04	0.837
19.5384 (2)	Chs8 (2)	Chitin synthase	2,551 (1,650–3,942)	1,267 (739–2,170)	6.32E-04	0.841
19.7214	Ipf885	Glucosidase	2,948 (1,260–6,898)	1,308 (675–2,534)	1.58E-03	0.764
19.4015	Cag1	α subunit of heterotrimeric G protein	7,808 (3,381–18,032)	4,380 (3,029–6,334)	1.58E-03	0.761
19.2946	Hnm4	Choline permease	3,001 (1,378–6,537)	1,251 (580–2,697)	1.76E-03	0.775
19.6861 (2)	Apc5 (2)	Subunit of anaphase-promoting complex	4,529 (2,365–8,674)	2,054 (1,022–4,129)	2.00E-03	0.793
19.3256 (2)	Sln1 (2)	Osmosensor	6,043 (1,635–22,332)	2,866 (1,695–4,846)	2.99E-03	0.639
19.6515	Hsp90	Heat shock protein	6,188 (1,263–30,307)	2,652 (1,481–4,748)	3.06E-03	0.620
19.3575	Cdc19	Pyruvate kinase	3,613 (1,033–12,639)	1,704 (960–3,024)	3.44E-03	0.706
19.7114 (2)	Csa1 (2)	Hemoglobin utilization	9,329 (4,016–21,671)	2,713 (792–9,293)	3.71E-03	0.798
19.3269 (2)	Gsl2 (2)	Glucan synthase	3,898 (2,725–5,575)	2,418 (1,561–3,746)	6.80E-03	0.806
19.4035	Pga4	Glucanosyltransferase	3,909 (2,496–6,124)	2,326 (1,297–4,170)	0.0203	0.774
19.2501 (2)	Flc1 (2)	Heme transporter	2,565 (1,564–4,208)	1,620 (1,004–2,615)	0.0220	0.774
19.7298 (2)	Chs1 (2)	Chitin synthase	2,504 (1,660–4,208)	1,529 (855–2,733)	0.0346	0.752

(Continued on next page)

TABLE 3 Antigenic biomarkers of early-convalescent-phase and mid-convalescent-phase candidemia patients *(Continued)*

Name		Description	Mean antigen reactivity (+/– SEM)		BH-adjusted *P* value	AUC
Systematic	Common		Candidemia patients	Negative controls		
19.4940	Ipf22247	Histidine permease	4,801 (2,852–8,081)	3,096 (1,887–5,078)	0.0391	0.730
19.2222	Yck22	Unknown function	4,387 (3,338–5,767)	2,913 (1,795–4,728)	0.0391	0.795
19.7313	Ssu1	Sulfite transporter	3,753 (2,853–4,936)	2,503 (1,533–4,085)	0.0405	0.775
19.1648 (1)	Rad50 (1)	DNA double-strand-break repair	6,226 (2,722–14,237)	3,323 (1,572–7,024)	0.0405	0.715
19.5148 (2)	Cyr1 (2)	Adenylyl cyclase	3,042 (2,240–4,130)	1,880 (1,002–3,526)	0.0478	0.747

[a]Reprinted from reference 19 with permission. AUC, area under the curve.

using a pXT7 linear vector (8) was employed for cloning PCR products of genes encoding cell surface proteins. For PCR amplification, all forward and reverse primers had common 33-nucleotide-long sequences at the 5′ end, followed by a gene-specific sequence (20 to 26 nucleotides). The common 33-nucleotide-long sequences at the 5′ end are homologous with the end sequences on the pXT7 linear vector. Each open reading frame was amplified from genomic DNA by using gene-specific primers. For genes larger than 3,000 kb, multiple sets of primers were employed to sequence segments of the gene to reduce the difficulty of PCR amplification.

In Vivo Recombination and In Vitro Transcription/Translation

To increase the proficiency of in vivo recombination, a previously reported high-efficiency (>90%) method was employed (8). Briefly, equal molar amounts of pTX7 linear vector and the PCR product were mixed and incubated with 50 μl of *Escherichia coli* DH5α competent cells on ice for 60 min in 96-well plates. After a 1-min heat shock at 42°C, the mixture was chilled on ice for 1 min and then incubated in 250 μl of SOC (super optimal catabolizer) medium (2% tryptone, 0.55% yeast extract, 10 mM NaCl, 10 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) for 1 h.

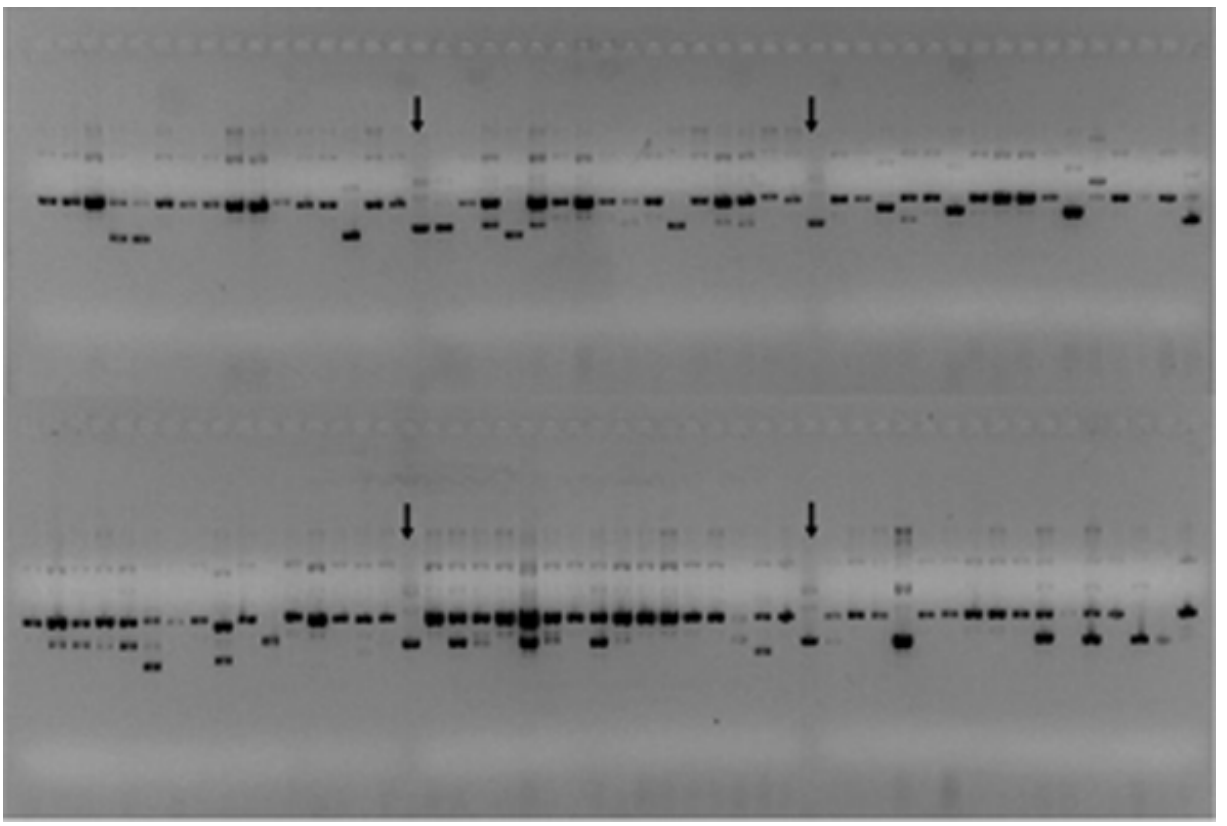

FIGURE 1 Verification of in vivo recombination. To confirm recombination of the pTX7 vector and PCR products, agarose gel electrophoresis was used. An empty vector (arrow) was used as a marker. Plasmids with *C. albicans* cell surface coding sequence inserts migrate more slowly than the empty vector. 10.1128/9781555817176.ch31f1

The resulting antibiotic-resistant cells were then grown overnight at 37°C in LB medium supplemented with 50 μg of kanamycin/ml. The plasmid was then isolated and purified without colony selection. The insertion success rate was confirmed using agarose gel electrophoresis (96.7%) (Fig. 1). The resulting circular plasmids with gene inserts were subjected to in vitro transcription/translation using an *E. coli*-based cell-free Rapid Translation System (RTS) 100 HY kit (Roche). In brief, the template DNA of the gene of interest is transcribed into mRNA by T7 RNA polymerase, which is then translated into protein by the ribosomal machinery in the *E. coli* lysate after 6 h of incubation at 30°C (Fig. 2). Following in vitro transcription/translation, each protein of interest will have been flanked by a hemagglutinin (HA) tag at the N terminus and 10× His tag at the C terminus. To assess the quantity and quality of the expressed proteins, Western blots were performed using mouse anti-poly-His monoclonal antibody (Sigma) followed by alkaline phosphatase-conjugated goat anti-mouse IgG (H+L) (Bio-Rad) or rat peroxidase-conjugated anti-HA antibody (Roche).

Protein Microarray Construction

The protein microarray was produced by printing the in vitro-expressed proteins in duplicate onto nitrocellulose-coated FAST glass slides (Schleicher & Schuell) at a density of 960 spots per slide using an automatic GMS417 robot (Genetic Microsystems) and the OmniGrid 100 (GeneMachines). The cell surface proteome contained 451 His- and/or HA-tagged peptides that represent 363 different proteins, as open reading frames of >3,000 bp were cloned into two or more segments. Proteins positive for HA tag and/or 10× His tag were immobilized on the array chips (HA positive, 84%; His positive, 61%). Proteins having at least one of the tags provided 98% coverage of the in vitro-expressed proteins. Each peptide was printed in duplicate and showed homogenous spot morphology as well as low background. The mean correlation between the duplicate printings of each peptide was 0.829. Internal controls consisting of buffer alone and a reaction mixture with no DNA were printed onto the FAST slides. For microarray detection, the proteome chips were blocked for 30 min with protein array blocking buffer (Schleicher & Schuell). Concurrently, a 1/50 dilution of human serum was incubated with 10% *E. coli* lysate in protein array blocking buffer for 30 min at room temperature. This step removes nonspecific antibodies directed against *E. coli* lysate in the cell-free in vitro transcription/translation system. The protein microarray was then probed with the human serum at 4°C overnight. The serum was aspirated and washed three times with Tris-buffered saline with 0.05% Tween 20 (TTBS). The protein array was then incubated with a biotin-conjugated donkey anti-human IgG Fc_γ fragment-specific secondary antibody (Jackson Immunoresearch) for 2 h at room temperature. The secondary antibody was then aspirated and washed three times with TTBS and then incubated with streptavidin:SureLight P-3 (Columbia Biosciences). GSI Lumonics scanned the hybridized slides,

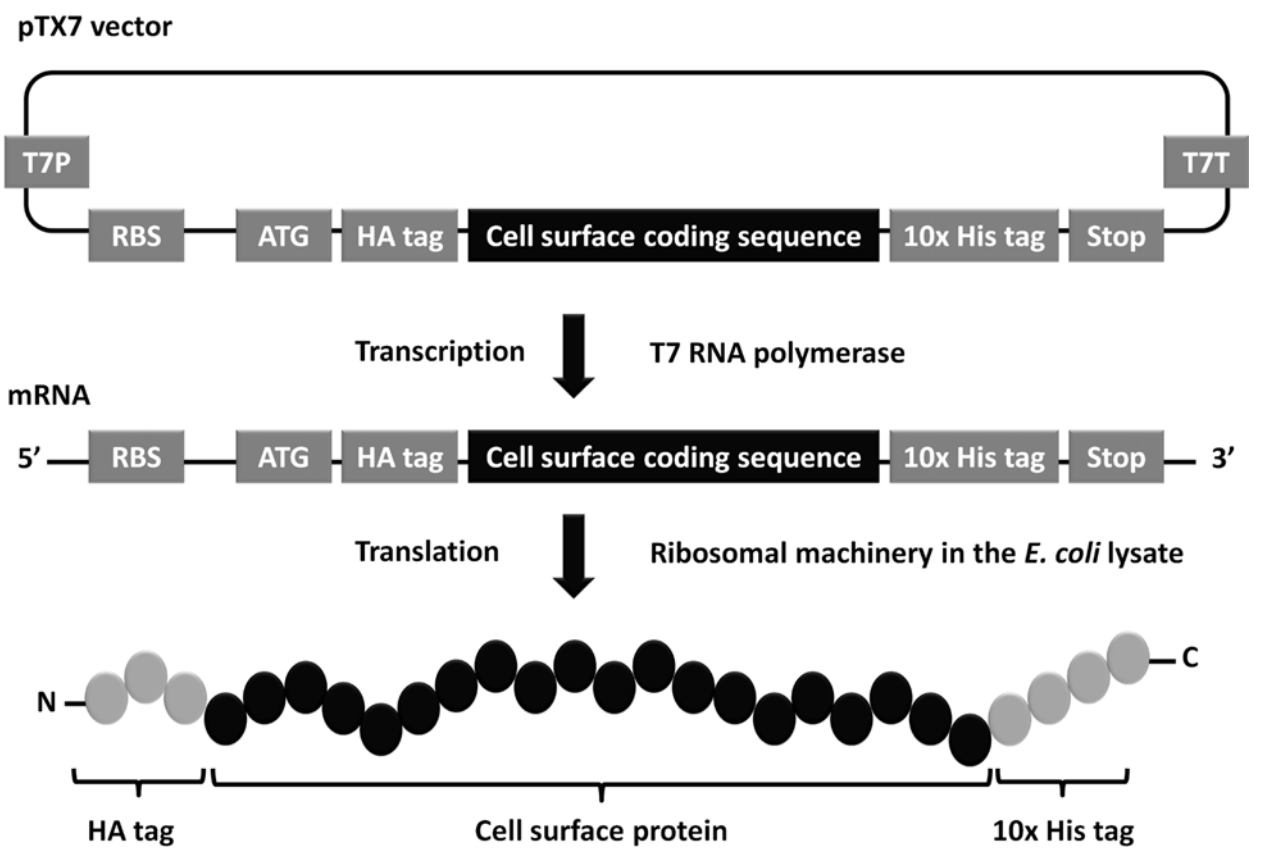

FIGURE 2 In vitro transcription/translation of *C. albicans* cell surface proteins. Template DNA of the gene of interest is transcribed into mRNA by T7 RNA polymerase, which is then translated into protein by the ribosomal machinery in the *E. coli* lysate after 6 h of incubation at 30°C. RBS, ribosomal binding site; T7P, T7 promoter; T7T, T7 terminator. 10.1128/9781555817176.ch31f2

and the intensity for each cell surface peptide was determined. Within each protein microarray, nine pairs of RTS reagents were printed and used as internal controls for nonspecific binding. The scanning of each patient was evaluated using a fixed titer of sera and was later adjusted using laser power and photomultiplier to obtain a relative level of intensity using spots without DNA and with buffer alone. Figure 3 shows a representative image of the microarray hybridized with the serum of an acute candidemia patient.

Statistical Analysis

All analysis was performed using the R statistical environment (http://www.r-project.org). It has been noted in the literature that data derived from microarray platforms is heteroskedastic (3, 10, 16). This mean-variance dependence has been observed in the arrays presented in this study (25, 26). In order to stabilize the variance, the variance stabilization and normalization (VSN) method (15) implemented as part of the Bioconductor suite (http://www.bioconductor.org) was applied to the quantified array intensities. In addition to removing heteroskedasticity, this procedure corrects for nonspecific noise effects by finding maximum-likelihood shifting and scaling parameters for each array such that the variances of a large number (default setting used, 85%) of the spots on the array are minimized. In other words, the method assumes that variances in binding for the vast

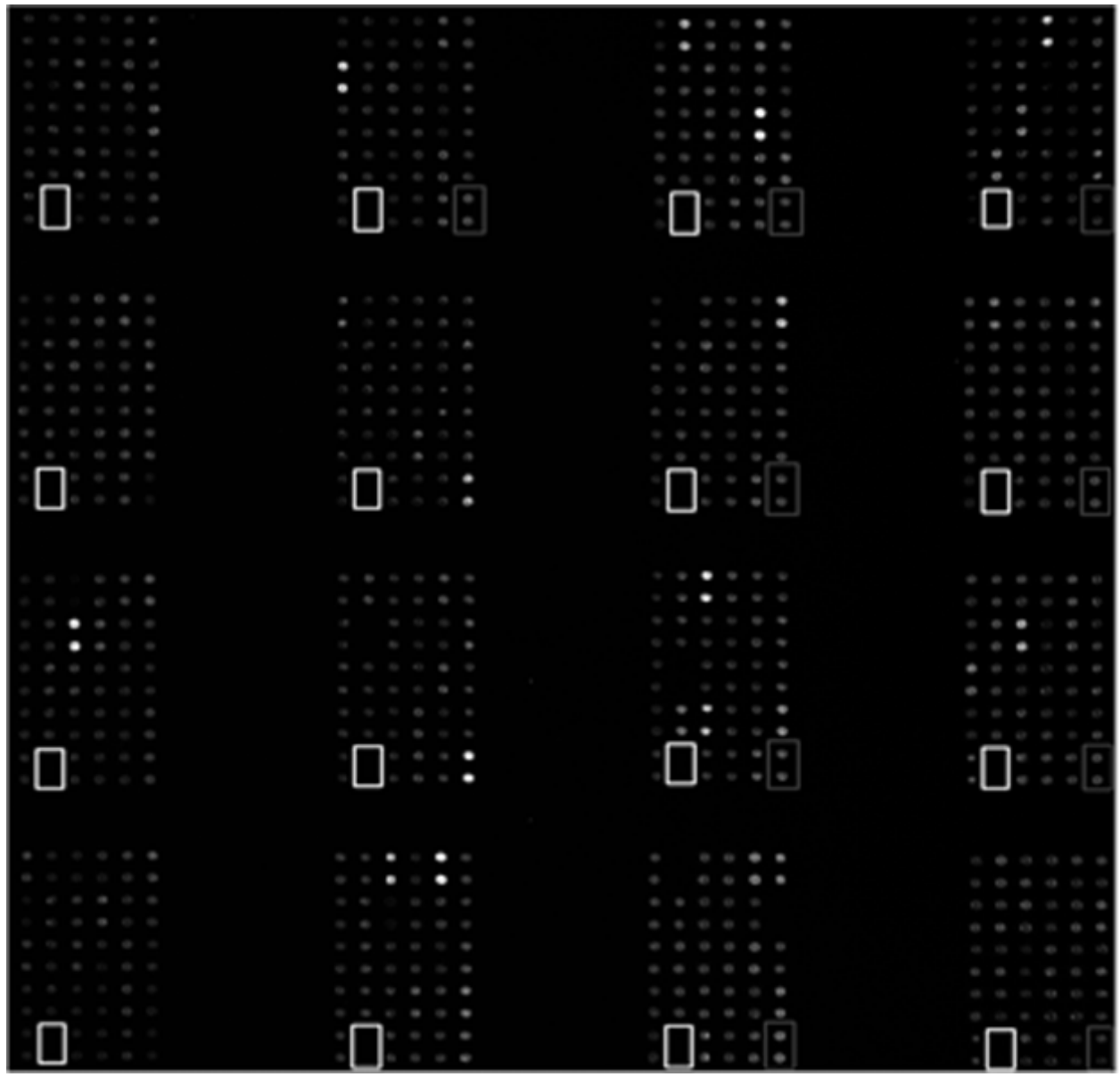

FIGURE 3 *Candida albicans* cell surface protein microarray. Shown is a representative image of the cell surface protein microarray of *C. albicans* hybridized with the sera of an acute candidemia patient. The array consisted of 16 subsets. Each of the *C. albicans* cell surface peptides was printed in duplicate. Each white box indicates a duplicated print of buffer alone, and each gray box shows a duplicate print of reaction mixture with no DNA. Reprinted from reference 19 with permission. 10.1128/9781555817176.ch31f3

majority of the proteins on the array are due to noise rather than true differential immunological response. In essence, 85% of the spots on the array are used as controls for sample-by-sample normalization. This calibration method has been shown to be effective on a number of platforms (4, 17, 24). A simple ranking normalization where all of the proteins are ordered for each sample by binding intensity and assigning the integer rank was performed as well, with similar results (results not shown). Finally, VSN-normalized data are retransformed with the "sinh" function to allow visualization and discussion at an approximate raw scale.

Diagnostic biomarkers between groups were determined using a Bayes regularized *t* test adapted from Cyber-T for protein arrays (2, 3). To account for multiple testing conditions, the Benjamini and Hochberg (BH) method was used to control the false discovery rate (14). Statistical analyses were performed with R 2.0 (http://www.r-project.org) and STATA (version 10.0; StataCorp). Multiple antigen classifiers were constructed using linear and nonlinear support vector machines using the "e1071" R package. To prevent overfitting and show the generalization of the classification method, 10 repeats of threefold cross-validation were performed. In this methodology, the data are split into three class-stratified subsets. For each subset, a classifier is trained using the remaining two-thirds of the data. The classifier is then evaluated on the one-third of the data not used for training. This process is repeated for each split and for 10 different splits, yielding 30 evaluation measures. The ROCR package was used to construct receiver-operating-characteristic curves and perform sensitivity and specificity analyses. Blast2Go (http://www.blast2go.org) was used for gene ontology annotation and enrichment analysis. To confirm that the identified antigens were accurate, their vectors were resequenced.

REFERENCES

1. **Alberti-Segui, C., A. J. Morales, H. Xing, M. M. Kessler, D. A. Willins, K. G. Weinstock, G. Cottarel, K. Fechtel, and B. Rogers.** 2004. Identification of potential cell-surface proteins in *Candida albicans* and investigation of the role of a putative cell-surface glycosidase in adhesion and virulence. *Yeast* **21:**285–302.
2. **Baldi, P., and S. R. Brunak.** 2001. *Bioinformatics: the Machine Learning Approach*. MIT Press, Cambridge, MA.
3. **Baldi, P., and A. D. Long.** 2001. A Bayesian framework for the analysis of microarray expression data: regularized t-test and statistical inferences of gene changes. *Bioinformatics* **17:**509–519.
4. **Barbacioru, C. C., Y. Wang, R. D. Canales, Y. A. Sun, D. N. Keys, F. Chan, K. A. Poulter, and R. R. Samaha.** 2006. Effect of various normalization methods on Applied Biosystems expression array system data. *BMC Bioinformatics* **7:**533.
5. **Braun, B. R., M. van Het Hoog, C. d'Enfert, M. Martchenko, J. Dungan, A. Kuo, D. O. Inglis, M. A. Uhl, H. Hogues, M. Berriman, M. Lorenz, A. Levitin, U. Oberholzer, C. Bachewich, D. Harcus, A. Marcil, D. Dignard, T. Iouk, R. Zito, L. Frangeul, F. Tekaia, K. Rutherford, E. Wang, C. A. Munro, S. Bates, N. A. Gow, L. L. Hoyer, G. Kohler, J. Morschhauser, G. Newport, S. Znaidi, M. Raymond, B. Turcotte, G. Sherlock, M. Costanzo, J. Ihmels, J. Berman, D. Sanglard, N. Agabian, A. P. Mitchell, A. D. Johnson, M. Whiteway, and A. Nantel.** 2005. A human-curated annotation of the *Candida albicans* genome. *PLoS Genet.* **1:**36–57.
6. **Brown, A. J., F. C. Odds, and N. A. Gow.** 2007. Infection-related gene expression in *Candida albicans*. *Curr. Opin. Microbiol.* **10:**307–313.
7. **Clancy, C. J., M. L. Nguyen, S. Cheng, H. Huang, G. Fan, R. A. Jaber, J. R. Wingard, C. Cline, and M. H. Nguyen.** 2008. Immunoglobulin G responses to a panel of *Candida albicans* antigens as accurate and early markers for the presence of systemic candidiasis. *J. Clin. Microbiol.* **46:**1647–1654.
8. **Davies, D. H., X. Liang, J. E. Hernandez, A. Randall, S. Hirst, Y. Mu, K. M. Romero, T. T. Nguyen, M. Kalantari-Dehaghi, S. Crotty, P. Baldi, L. P. Villarreal, and P. L. Felgner.** 2005. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc. Natl. Acad. Sci. USA* **102:**547–552.
9. **De Groot, P. W., K. J. Hellingwerf, and F. M. Klis.** 2003. Genome-wide identification of fungal GPI proteins. *Yeast* **20:**781–796.
10. **Durbin, B. P., J. S. Hardin, D. M. Hawkins, and D. M. Rocke.** 2002. A variance-stabilizing transformation for gene-expression microarray data. *Bioinformatics* **18**(Suppl. 1)**:**S105–S110.
11. **Fan, J., V. Chaturvedi, and S. H. Shen.** 2002. Identification and phylogenetic analysis of a glucose transporter gene family from the human pathogenic yeast *Candida albicans*. *J. Mol. Evol.* **55:**336–346.
12. **Han, Y., M. H. Riesselman, and J. E. Cutler.** 2000. Protection against candidiasis by an immunoglobulin G3 (IgG3) monoclonal antibody specific for the same mannotriose as an IgM protective antibody. *Infect. Immun.* **68:**1649–1654.
13. **Han, Y., M. A. Ulrich, and J. E. Cutler.** 1999. *Candida albicans* mannan extract-protein conjugates induce a protective immune response against experimental candidiasis. *J. Infect. Dis.* **179:**1477–1484.
14. **Hochberg, Y., and Y. Benjamini.** 1990. More powerful procedures for multiple significance testing. *Stat. Med.* **9:**811–818.
15. **Huber, W., A. von Heydebreck, H. Sultmann, A. Poustka, and M. Vingron.** 2002. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* **18**(Suppl. 1)**:**S96–S104.
16. **Ideker, T., V. Thorsson, A. F. Siegel, and L. E. Hood.** 2000. Testing for differentially-expressed genes by maximum-likelihood analysis of microarray data. *J. Comput. Biol.* **7:**805–817.
17. **Kreil, D. P., N. A. Karp, and K. S. Lilley.** 2004. DNA microarray normalization methods can remove bias from differential protein expression analysis of 2D difference gel electrophoresis results. *Bioinformatics* **20:**2026–2034.
18. **Matthews, R. C., G. Rigg, S. Hodgetts, T. Carter, C. Chapman, C. Gregory, C. Illidge, and J. Burnie.** 2003. Preclinical assessment of the efficacy of Mycograb, a human recombinant antibody against fungal HSP90. *Antimicrob. Agents Chemother.* **47:**2208–2216.
19. **Mochon, A. B., J. Ye, M. A. Kayala, J. R. Wingard, C. J. Clancy, M. H. Nguyen, P. Felgner, P. Baldi, and H. Liu.** 2010. Serological profiling of a *Candida albicans* protein microarray reveals permanent host-pathogen interplay and stage-specific responses during candidemia. *PLoS Pathog.* **6:**e1000827.
20. **Monteoliva, L., M. L. Matas, C. Gil, C. Nombela, and J. Pla.** 2002. Large-scale identification of putative exported proteins in *Candida albicans* by genetic selection. *Eukaryot. Cell* **1:**514–525.
21. **Pachl, J., P. Svoboda, F. Jacobs, K. Vandewoude, B. van der Hoven, P. Spronk, G. Masterson, M. Malbrain, M. Aoun, J. Garbino, J. Takala, L. Drgona, J. Burnie, and R. Matthews.** 2006. A randomized, blinded, multicenter trial of lipid-associated amphotericin B alone versus in

combination with an antibody-based inhibitor of heat shock protein 90 in patients with invasive candidiasis. *Clin. Infect. Dis.* **42:**1404–1413.

22. **Pitarch, A., J. Abian, M. Carrascal, M. Sanchez, C. Nombela, and C. Gil.** 2004. Proteomics-based identification of novel *Candida albicans* antigens for diagnosis of systemic candidiasis in patients with underlying hematological malignancies. *Proteomics* **4:**3084–3106.
23. **Pitarch, A., A. Jimenez, C. Nombela, and C. Gil.** 2006. Decoding serological response to *Candida* cell wall immunome into novel diagnostic, prognostic, and therapeutic candidates for systemic candidiasis by proteomic and bioinformatic analyses. *Mol. Cell. Proteomics* **5:**79–96.
24. **Sarkar, D., R. Parkin, S. Wyman, A. Bendoraite, C. Sather, J. Delrow, A. K. Godwin, C. Drescher, W. Huber, R. Gentleman, and M. Tewari.** 2009. Quality assessment and data analysis for microRNA expression arrays. *Nucleic Acids Res.* **37:**e17.
25. **Sundaresh, S., D. L. Doolan, S. Hirst, Y. Mu, B. Unal, D. H. Davies, P. L. Felgner, and P. Baldi.** 2006. Identification of humoral immune responses in protein microarrays using DNA microarray data analysis techniques. *Bioinformatics* **22:**1760–1766.
26. **Sundaresh, S., A. Randall, B. Unal, J. M. Petersen, J. T. Belisle, M. Gill Hartley, M. Duffield, R. W. Titball, D. H. Davies, P. L. Felgner, and P. Baldi.** 2007. From protein microarrays to diagnostic antigen discovery: a study of the pathogen *Francisella tularensis*. *Bioinformatics* **23:**i508–i518.
27. **Urban, C., K. Sohn, F. Lottspeich, H. Brunner, and S. Rupp.** 2003. Identification of cell surface determinants in *Candida albicans* reveals Tsa1p, a protein differentially localized in the cell. *FEBS Lett.* **544:**228–235.

Candida *and Candidiasis*, 2nd Edition
Edited by R. A. Calderone and C. J. Clancy

10.1128/9781555817176.ch32

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Cool Tools 3: Large-Scale Genetic Interaction Screening in *Candida albicans*

YEISSA CHABRIER-ROSELLÓ, ANUJ KUMAR, AND DAMIAN J. KRYSAN

Although the genetic study of *Candida albicans* is generally less convenient than that of *Saccharomyces cerevisiae*, recent advances in the molecular genetics of *C. albicans* have greatly facilitated the study of this important human fungal pathogen. In terms of genetic study, the key difference between *C. albicans* and *S. cerevisiae* is that *S. cerevisiae* has a complete sexual cycle, whereas *C. albicans* is an obligate diploid organism without a classical sexual cycle. Instead, *C. albicans* exhibits a parasexual cycle in which **a** and α diploid cells mate to generate an **a**/α tetraploid strain under specific environmental conditions. The tetraploid cells then resolve to euploidy by random chromosome loss (1, 9, 15). For the study of single genes, the obligate diploid status of *C. albicans* means that in general, two mutations must be created in order to study a null mutation; recent advances in genetic engineering of *C. albicans* have made such strain constructions a relatively straightforward procedure. However, the lack of a classical sexual cycle renders the study of genetic interactions, the effect of mutations at two separate loci, much more problematic in *C. albicans* than in *S. cerevisiae*. In this chapter we discuss the development of a novel strategy for large-scale synthetic genetic analysis in *C. albicans* based on complex haploinsufficiency (CHI).

A synthetic genetic interaction (also called negative or aggravating interaction) is a type of genetic interaction that occurs when a strain containing mutations at two distinct loci exhibits a phenotype (synthetic sickness or lethality) that is more severe than that of either single mutation alone (6, 19). In *S. cerevisiae*, mating-based strategies allow the rapid, systematic construction of sets of double mutants consisting of a mutation of interest (the query mutation) combined with the entire set of ~4,800 non-essential single gene deletion mutations in *S. cerevisiae* (6, 10, 13): the so-called synthetic genetic array. The genome-wide study of synthetic genetic interactions has greatly facilitated the understanding, identification, and mapping of biochemical pathways and complexes in the model yeast *S. cerevisiae* (6, 10). Through these studies, it has become apparent that synthetic genetic interactions cluster within functional groups and, many times, represent genes that function in parallel. It has also been shown that strains containing random pairs of deleterious mutations rarely display synthetic growth defects (14), further supporting the likelihood that mechanistically relevant information will be generated through synthetic genetic analysis of a given cellular process.

Synthetic genetic analysis has been applied to *C. albicans* in a gene-by-gene fashion, i.e., to study filamentation control (2, 3). For example, Braun and Johnson demonstrated a synthetic genetic interaction between *TUP1* and *CPH1*, and *EFG1* and *CPH1*, where *tup1*Δ/Δ is epistatic to *cph1*Δ/Δ and *efg1*Δ/Δ is epistatic to *cph1*Δ/Δ. From these data they clearly showed that *TUP1*, *EFG1*, and *CPH1* make separate contributions to the regulation of filamentation in *C. albicans* (3, 7, 10, 11, 16). Although *C. albicans* synthetic genetic studies have been limited to gene-by-gene analyses, the power of these studies is clearly apparent and suggests that large-scale studies could yield important insights into the biology of *C. albicans*.

Large-scale, single-mutant genetic screens employing transposon-based mutagenesis to study gene function have been carried out in *C. albicans* (4). In general, two types of large-scale, transposon-based genetic screens have been reported in *C. albicans* (5, 8, 21). In the first type, large libraries of homozygous mutants have been generated using a transposon containing the *UAU1* cassette (*URA3-ARG4-URA3*). Homozygous mutants, arising from an initial transposon insertion followed by a gene conversion event, are selected by growth on medium lacking both uridine and arginine. For example, Davis et al. utilized an insertional-mutagenesis strategy based on the *UAU1* cassette in a large-scale genetic screen to identify genes involved in pH-dependent filamentation (5).

The second type of large-scale genetic screens that have been performed in *C. albicans* is based on the concept of haploinsufficiency. Haploinsufficiency refers to the case

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where mutation of one allele of a given gene in a diploid organism results in a measurable phenotype since the single functional copy of the gene does not produce enough of the gene product (6) (Fig. 1). In the seminal study by Uhl et al. (21), the authors used simple haploinsufficiency to identify 146 genes involved in this morphological transition. Among these were genes involved in nutrient sensing, cytoskeletal organization, cell wall construction, and transcriptional control. In addition, large-scale genetic screens involving chemically induced haploinsufficiency have been used to discover and study the mechanisms of action of novel antifungal molecules.

Haploinsufficiency-based genetic studies have also been used in *S. cerevisiae* to identify pairs of interacting genes. This was first applied to the study of cytoskeletal genes such as tubulin and actin genes (12, 20, 22). In this approach, double heterozygote mutants containing heterozygous mutations in an essential gene of interest and at a second locus were generated and screened for strains that retain the phenotype of the parental strain. This genetic phenomenon is called unlinked noncomplementation.

Haarer et al. identified a large set of actin interacting genes through the use of a special case of unlinked noncomplementation termed CHI (12). CHI is manifested when a strain with two heterozygous mutations at separate loci displays a phenotype more severe than either of the single heterozygous mutants. As an illustration of the concept, an actin null mutant (*act1*Δ) containing *ACT1* on a plasmid was mated to the set of single-gene-deletion strains and then cured of the plasmid to give a set of complex heterozygotes (*act1*Δ/*ACT1 yfg*Δ/*YFG*). Many of the genes identified using this genetic approach demonstrated defects in the actin cytoskeleton, confirming the ability of CHI to identify interacting pairs of genes and genes with similar cellular functions.

Here we describe the development of a large-scale synthetic genetic screen using CHI to understand the role of the RAM (regulation of Ace2 and morphogenesis) network in *C. albicans* morphological transition. The RAM network primarily regulates the transcription factor Ace2p, which is required for virulence (17), through the serine/threonine protein kinase Cbk1p. In our genetic approach (Fig. 2) a strain containing a heterozygous null mutation of interest (*cbk1*Δ/*CBK1*) (18) was subjected to transposon-mediated mutagenesis to generate a large set of double mutant strains containing the initial query mutation (*cbk1*Δ/*CBK1*) and a transposon-derived mutation at the second locus.

We constructed a modified Tn*7* transposon containing the recyclable *C. albicans URA3* auxotrophic marker *URA3-dpl200* cassette in the Tn*7* transposon donor plasmid pGPS3. The resulting transposon was then used to mutagenize a genomic library of *C. albicans* strain WO-1. The Tn*7*-mutagenized inserts contained in the resulting library were released from the acceptor plasmid by digestion with PvuII, and the resulting linear DNA was transformed into the Ura$^-$, *cbk1*Δ/*CBK1* strain derived from CAI4 to give 6,528 independent Ura$^+$ transformants. To identify double heterozygous mutants displaying CHI with *cbk1*Δ/*CBK1*, mutants were spotted onto Spider medium and incubated at 37°C. Colonies with few or no peripheral hyphae as well as a smooth colony appearance were scored as having CHI with *cbk1*Δ/*CBK1*.

To differentiate between mutations exhibiting synthetic genetic interactions with *CBK1* from mutations causing phenotypes solely due to being transposon associated, a wild-type allele of *CBK1* was reintegrated into the double heterozygotes following 5-fluoro-orotic acid-mediated recombinational excision of the *URA3* marker. If the transposon-associated mutation causes a very severe hyphal phenotype by itself, then restoration of *CBK1* homozygosity will not alter the phenotype of the double mutant significantly. On the other hand, if the double-mutant phenotype is due to an interaction between *cbk1*Δ and the transposon-derived mutation, then reintroduction of the wild-type *CBK1* allele will lessen the hyphal growth defects. From a set of 42 candidate interacting genes identified by our screen, 41 were found to be true interactors. It is likely that this low false-positivity rate is due to the fact that we started with a parental strain (*cbk1*Δ/*CBK1*) that was itself haploinsufficient; consequently, the effects of noninteracting mutations that display simple haploinsufficiency were masked by the phenotype of the parental strain.

In principle, CHI-based screening will identify three classes of interacting genes. First, genes that function within the same pathway (RAM) as the query gene (*CBK1*) will be identified if partial loss of activity in two components of the multicomponent process/pathway leads to a synergistic or additive loss of function. A second class of interacting genes that may emerge from a CHI screen is genes that function in parallel with the parental mutation. The third possible type

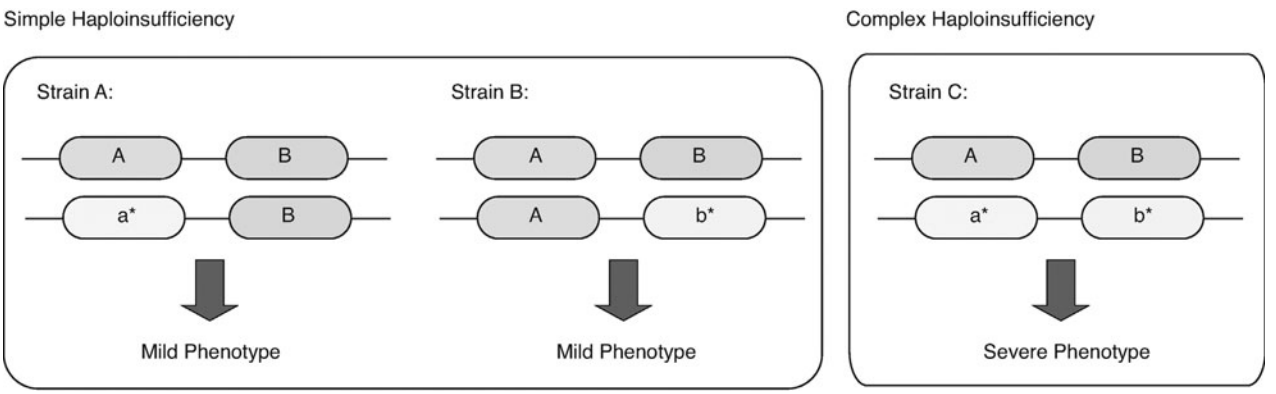

FIGURE 1 Schematic depiction of simple haploinsufficiency and CHI. a* and b*, mutation in one allele of strain A or B in diploid *C. albicans*. 10.1128/9781555817176.ch32f1

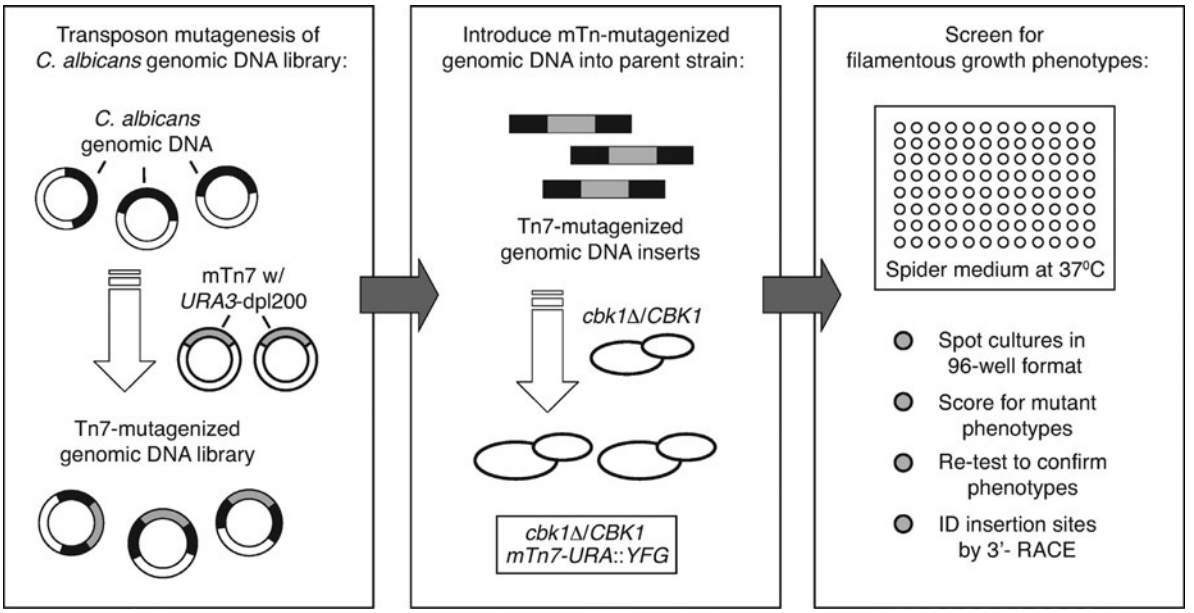

FIGURE 2 Schematic of screening strategy with in vitro mutagenesis of C. *albicans* genomic library WO-1 using a Tn*7*-based transposon containing the CaURA3-dpl200 auxotrophic marker. The resulting library was screened on Spider medium for altered filamentation relative to the parental strain. 10.1128/9781555817176.ch32f2

of interacting genes likely to be identified is a set that simply decreases the general fitness of the strain and thereby decreases its ability to carry out a given cellular function (i.e., hyphal development). An important feature of the CHI-based approach is that, unlike homozygous mutant-based strategies, genetic interactions involving essential genes can be detected.

The set of 41 genes identified in this screen was enriched in transcriptional targets of Ace2 and, strikingly, the cyclic AMP-dependent protein kinase A (PKA) pathway, which suggested an interaction between these two pathways (Fig. 3). We found that 88% of the genes found through this screen were Ace2 regulated, 46% were PKA regulated, and 34% were Ace2 and PKA coregulated. Taking these results all together, we were able to identify two different classes of genes mentioned above: genes functioning within the same pathway (RAM) and genes interacting with a possible parallel pathway (PKA) during morphogenesis.

The success of our screen, the first large-scale genetic interaction screen performed in C. *albicans*, strongly suggests that this approach should be generally applicable to the study of this important organism. As new mutagenesis techniques are developed and as large collections of mutants are created, it is likely that the creative application of these principles to genetic interaction analysis will lead to a deeper understanding of the complex regulatory networks that orchestrate C. *albicans* biology and pathogenesis.

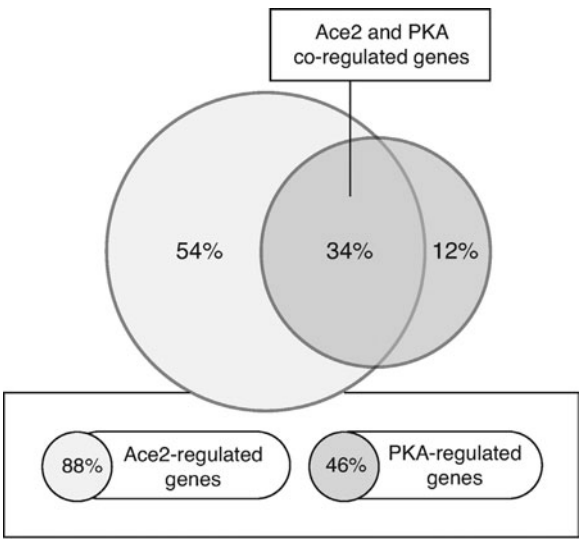

FIGURE 3 Venn diagram demonstrating the percentage of genes coregulated by RAM, PKA, or both pathways found through our CHI-based screen. 10.1128/9781555817176.ch32f3

REFERENCES

1. **Bennett, R. J., and A. D. Johnson.** 2003. Completion of a parasexual cycle in *Candida albicans* by induced chromosome loss in tetraploid strains. *EMBO J.* **22:**2505–2515.
2. **Braun, B. R., and A. D. Johnson.** 1997. Control of filament formation in *Candida albicans* by the transcriptional repressor TUP1. *Science* **277:**105–109.
3. **Braun, B. R., and A. D. Johnson.** 2000. TUP1, CPH1 and EFG1 make independent contributions to filamentation in *Candida albicans*. *Genetics* **155:**57–67.
4. **Bruno, V. M., and A. P. Mitchell.** 2004. Large-scale gene function analysis in *Candida albicans*. *Trends Microbiol.* **12:**157–161.
5. **Davis, D. A., V. M. Bruno, L. Loza, S. G. Filler, and A. P. Mitchell.** 2002. *Candida albicans* Mds3p, a conserved regulator of pH responses and virulence identified through insertional mutagenesis. *Genetics* **162:**1573–1581.

6. **Dixon, S. J., M. Costanzo, A. Baryshnikova, B. Andrews, and C. Boone.** 2009. Systematic mapping of genetic interaction networks. *Annu. Rev. Genet.* **43:**601–625.
7. **Doedt, T., S. Krishnamurthy, D. P. Bockmühl, B. Tebarth, C. Stempel, C. L. Russell, A. J. Brown, and J. F. Ernst.** 2004. APSES proteins regulate morphogenesis and metabolism *in Candida albicans*. *Mol. Biol. Cell* **15:**3167–3180.
8. **Enloe, B., A. Diamond, and A. P. Mitchell.** 2000. A single-transformation gene function test in diploid *Candida albicans*. *J. Bacteriol.* **182:**5730–5736.
9. **Forche, A., K. Alby, D. Schaefer, A. D. Johnson, J. Berman, and R. J. Bennett.** 2008. The parasexual cycle in *Candida albicans* provides an alternative pathway to meiosis for the formation of recombinant strains. *PLoS Biol.* **6:**e110.
10. **Giaever, G., A. M. Chu, L. Ni, C. Connelly, L. Riles, et al.** 2002. Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418:**387–391.
11. **Giusani, A. D., M. Vinces, and C. A. Kumamoto.** 2002. Invasive filamentous growth of *Candida albicans* is promoted by Czf1p-dependent relief of Efg1p-mediated repression. *Genetics* **160:**1749–1753.
12. **Haarer, B., S. Viggiano, M. A. Hibbs, O. G. Troyanskaya, and D. C. Amberg.** 2007. Modeling complex genetic interactions in a simple eukaryotic genome: actin displays a rich spectrum of complex haploinsufficiencies. *Genes Dev.* **21:**148–159.
13. **Harrison, R., B. Papp, C. Pál, S. G. Oliver, and D. Delneri.** 2007. Plasticity of genetic interactions in metabolic networks of yeast. *Proc. Natl. Acad. Sci. USA* **104:**2307–2312.
14. **Hartman, J. L., IV, B. Garvik, and L. Hartwell.** 2001. Principles for the buffering of genetic variation. *Science* **291:**1001–1004.
15. **Hull, C. M., R. M. Raisner, and A. D. Johnson.** 2000. Evidence for mating of the "asexual" yeast *Candida albicans* in a mammalian host. *Science* **289:**256–257.
16. **Kebaara, B. W., M. L. Langford, D. H. Navarathna, R. Dumitru, K. W. Nickerson, and A. L. Atkin.** 2008. *Candida albicans* Tup1 is involved in farnesol-mediated inhibition of filamentous-growth induction. *Eukaryot. Cell* **7:**980–987.
17. **Kelly, M. T., D. M. MacCallum, S. D. Clancy, F. C. Odds, A. J. Brown, and G. Butler.** 2004. The *Candida albicans* CaACE2 gene affects morphogenesis, adherence and virulence. *Mol. Microbiol.* **53:**969–983.
18. **McNemar, M. D., and W. A. Fonzi.** 2002. Conserved serine/threonine kinase encoded by CBK1 regulates expression of several hypha-associated transcripts and genes encoding cell wall proteins in *Candida albicans*. *J. Bacteriol.* **184:**2058–2061.
19. **Nijman, S. M.** 2011. Synthetic lethality: general principles, utility and detection using genetic screens in human cells. *FEBS Lett.* **585:**1–6.
20. **Stearns, T., and D. Botstein.** 1988. Unlinked noncomplementation: isolation of new conditional-lethal mutations in each of the tubulin genes of *Saccharomyces cerevisiae*. *Genetics* **119:**249–260.
21. **Uhl, M. A., M. Biery, N. Craig, and A. D. Johnson.** 2003. Haploinsufficiency-based large-scale forward genetic analysis of filamentous growth in the diploid human fungal pathogen *C. albicans*. *EMBO J.* **22:**2668–2678.
22. **Vinh, D. B., M. D. Welch, A. K. Corsi, K. F. Wertman, and D. G. Drubin.** 1993. Genetic evidence for functional interactions between actin noncomplementing (Anc) gene products and actin cytoskeletal proteins in *Saccharomyces cerevisiae*. *Genetics* **135:**275–286.

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10.1128/9781555817176.ch33

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Cool Tools 4: Imaging *Candida* Infections in the Live Host

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The host response to *Candida* is a critical determinant in whether the organism is maintained in a commensal state, cleared after inadvertent introduction to tissues, or allowed to cause local or disseminated disease. Thus, understanding the interactions between *Candida* and the host is a key step to developing strategies to prevent or treat *Candida* disease. Using a combination of animal model systems and in vitro studies, *Candida* researchers have made substantial progress investigating these interactions. However, as with all model systems, each of the traditional models of host-*Candida* interactions has limitations that prevent us from fully understanding how *Candida* and the host interact.

Studies investigating the interaction of *Candida* with a single host cell type such as phagocytes or epithelial cells are typically performed in vitro (3, 14, 25). The advantages of in vitro studies include the ability to compare multiple conditions in one experiment, the ability to perform thorough biochemical and immunologic analyses, the relatively short duration of experiments, lower experimental costs, and the ability to reduce the use of laboratory animals. The major disadvantage of in vitro experiments is that it is not possible to fully replicate the myriad of cellular and extracellular signals provided by the host environment. Signals from the extracellular environment substantially alter the response of the host cell. For example, neutrophil adhesion, migration, spreading, and phagocytosis are influenced by the substrate on which neutrophils are plated (5, 6). In typical in vitro experiments, the substrate is plastic or glass, which clearly does not mimic the three-dimensional extracellular matrix that occurs in the host. Thus, the simplicity of in vitro model systems is both their major advantage and disadvantage.

Several animal models of candidiasis are regularly used, including models of oropharyngeal, vaginal, and disseminated candidiasis (18, 20, 24, 26). The advantage of these models is that they allow us to explore *Candida* pathogenesis in an intact host. Typical outcome measurements for in vivo models of candidiasis include survival and organism burden. Information about the interaction of specific host cell types with *Candida* organisms in vivo can sometimes be obtained through conventional histology or immunohistochemistry, but these analyses require sacrifice of the animal and thus are often limited to a single time point during the course of infection. Because of the reliance on survival as an endpoint for models of systemic candidiasis, it is difficult to use the model to study how the intact immune system successfully defends the host against *C. albicans* disease. Recently, luminescent strains of *Candida* have been used in conjunction with whole-mouse in vivo imaging systems (IVIS systems; Caliper Life Sciences) to monitor the course of superficial and subcutaneous infections (9, 10, 22). This system produces images of the whole mouse overlaid with a map of luminescence intensity to monitor organism burden over time. Unfortunately, it has not yet been successful for the study of systemic disease. While IVIS systems are excellent for following organism burden over time, they do not provide specific information about host-organism interactions.

In contrast, our goal was to develop a model in which we could obtain high-spatial-resolution information about the host-*Candida* interaction within native host tissues without sacrificing the mouse. The recent expansion of fluorescence in vivo imaging techniques, including confocal and multiphoton microscopy, has enabled acquisition of high-resolution images of resident dendritic cells as well as malaria and leishmania infections and responding T cells in the pinnae (ear flaps) of live mice (1, 8, 12, 21). Using a similar system, we injected fluorescent-protein-expressing *Candida* intradermally into the pinnae of anesthetized mice to obtain high-spatial-resolution images of *Candida* within native host tissue (19). This system allows us to obtain images serially over time, and thus, we can readily monitor both severe infections that go on to produce disseminated disease and infections that are resolved by the host. With injections of $\geq 10^5$ CFU of *Candida*, mice develop significant local inflammation with swelling and redness at the injection site. Injection of relatively high doses of *Candida* (approximately 10^7 CFU) leads to dissemination and the development of systemic candidiasis. Thus, this technique

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produces a realistic model of tissue infection with local inflammation and potential dissemination.

There have been two reports describing the use of reflectance confocal microscopy to image the cornea in patients or horses with *Candida* keratitis that visualized reflective linear or elongated structures (4, 17); however, these studies did not definitively identify the structures as fungal elements. Furthermore, these studies were performed as diagnostic studies; they were not designed to monitor or manipulate the infection. Thus, the *Candida* in vivo imaging model system described here represents the first time we have been able to obtain high-resolution images of experimental *Candida* host-pathogen interactions in the tissue of a living host.

METHOD

Candida

Several fluorescent proteins, including yeast-enhanced green fluorescent protein (yeast EGFP) and mCherry (yEmRFP), have recently been codon optimized for expression in yeast (7, 13, 16). We suspect that any strain of *Candida* that has relatively bright fluorescence could be used in this model. We have had good success using YAW3, a BWP17 strain in which yeast EGFP is expressed under the control of the constitutively active ADH1 promoter (generously provided by James Konopka, Stonybrook University), and SKY38, a BWP17 strain with PADH1-yEmRFP-URA3-ARS (16). Both of these strains have had random integration of the fluorescent protein into the *Candida* genome and demonstrate bright cytoplasmic fluorescence with uniform fluorescence throughout yeast and filamentous forms.

To prepare yeast for injection, overnight cultures are passaged daily for 3 days, after which yeasts are washed three times, counted in a hemocytometer, and adjusted to the desired organism density in endotoxin-free phosphate-buffered saline.

Mice

We have successfully used this model with mice of various strain backgrounds, including white-, brown-, and black-haired mice. In order to minimize chlorophyll-derived autofluorescence, mice are fed a chlorophyll-free chow for at least a week prior to imaging (15). If desired, immunocompromised mice, such as DBA/2N mice, which cannot recruit neutrophils to the site of *Candida* infection (23), can be used to explore the effect of immunocompromise on in vivo *Candida* infections. Alternatively, mice with fluorescently labeled host cells, such as the LysM-GFP mice, in which neutrophils and macrophages express GFP, can be used to obtain information about the interaction of *Candida* with specific cell types (11).

Inoculation

Prior to inoculation, mice are anesthetized with ketamine/xylazine and given artificial tears to maintain eye moisture. Hair is removed from the pinna by applying a commercial depilatory such as Nair (Church and Dwight Company, Inc.) for 2 to 3 min and removing it by several washes with sterile water and moistened cotton swabs. Mice are then inoculated intradermally using a 0.3-ml insulin syringe, 29-gauge, ½-in. needle to inject a 10- to 20-μl volume of *Candida* in phosphate-buffered saline.

Imaging

For imaging, an inverted confocal microscope with an enclosed stage incubator chamber and generic stage insert is used. If a generic stage insert is not available, it may be necessary to construct a stage insert that allows placement of the coverslips and mouse. Our initial experiments were performed using a custom-built inverted confocal microscope (2). More recently, we have constructed a stage insert that allows us to use an Olympus FV1000 laser scanning confocal microscope at our confocal microscopy core facility. We have successfully obtained images using both 10×, 0.4- to 0.45-numerical-aperture (NA) and 20×, 0.75-NA dry objective lenses.

A coverslip is placed on the stage over the opening for the objective and taped in place. Next, the anesthetized mouse is placed on the stage so that the pinna of the injected ear is centered on the coverslip over the objective opening. The pinna is then flattened as much as possible by placing a second coverslip over the ear and taping the second coverslip in place. In this way, the ear is restrained between the two coverslips (Fig. 1). As no restraints other than the coverslips are placed on the mouse, it is important to monitor sedation throughout the imaging period. The mouse is kept warm by setting the stage warming chamber to 37°C. If a stage warming chamber is not available, a thermal blanket can be used.

Imaging then proceeds as might be done with a tissue section. It is often necessary to scan through multiple fields to find an area with good distribution of *Candida*. Once such an area is located, we have found it helpful to obtain a *z*-stack of images, including bright-field or differential interference contrast images and images of the relevant fluorescence wavelength(s), to identify *Candida* in the various tissue planes. When imaging is completed, the mouse is allowed to recover in a warmed cage and can be sedated and imaged again as needed.

SUMMARY

The relatively large size of *Candida* and our current ability to achieve strong expression of a variety of fluorescent

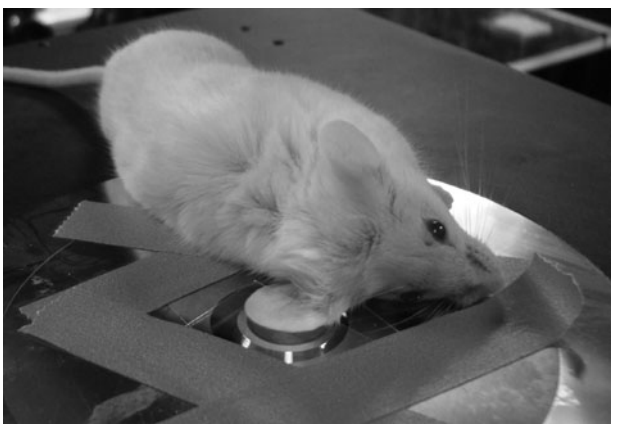


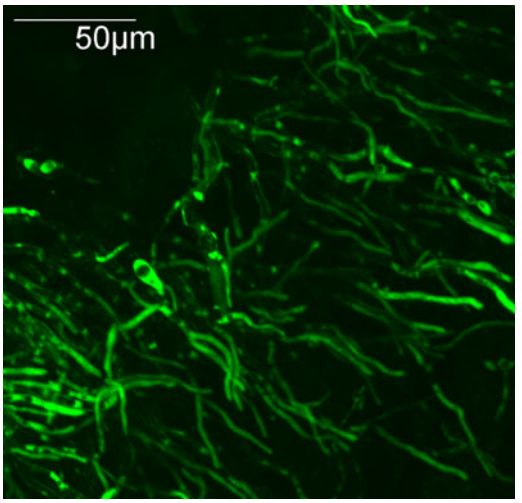
FIGURE 1 Immobilization of the pinna. A coverslip is secured over the opening for the objective lens using tape. An anesthetized mouse is then laid on the stage with the pinna centered over the objective lens. A second coverslip, which is used to flatten and restrain the pinna, is also secured with tape. It should be noted that the mouse is not taped down—the tape simply holds the coverslips in place. 10.1128/9781555817176.ch33f1

proteins lend themselves to an in vivo imaging model system. With this system, we have successfully obtained high-spatial-resolution images of *Candida* growing in the native host environment. The resolution obtained with these images is sufficient to visualize large yeast structures such as the vacuole and buds and to readily discriminate yeast and filamentous forms (Color Plate 6). We have also been able to obtain images, like that shown in Color Plate 7, of GFP-labeled phagocytes infiltrating into the area of *Candida* infection within 4 h after inoculation. In the future, we hope to use this model to explore more details about host and pathogen factors that interact to control the inflammatory response to the organism and that contribute to or prevent dissemination into tissues.

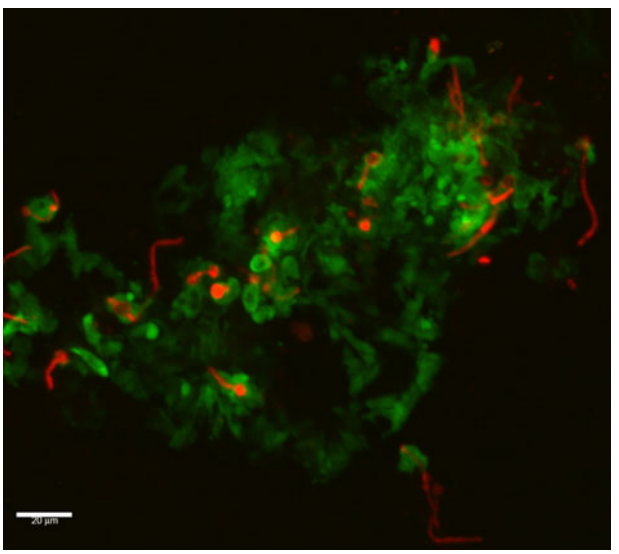
This work was funded in part by the University of Rochester Clinical and Translational Sciences Institute (NIH National Center for Research Resources [ULI RR024160-1]) and National Cancer Institute grants CA68409 and CA122093.

REFERENCES

1. **Amino, R., S. Thiberge, S. Blazquez, P. Baldacci, O. Renaud, S. Shorte, and R. Menard.** 2007. Imaging malaria sporozoites in the dermis of the mammalian host. *Nat. Protoc.* **2:**1705–1712.
2. **Bigelow, C. E., D. L. Conover, and T. H. Foster.** 2003. Confocal fluorescence spectroscopy and anisotropy imaging system. *Opt. Lett.* **28:**695–697.
3. **Bourgeois, C. M., O. Majer, I. Frohner, and K. Kuchler.** 2009. In vitro systems for studying the interaction of fungal pathogens with primary cells from the mammalian innate immune system. *Methods Mol. Biol.* **470:**125–139.
4. **Brasnu, E., T. Bourcier, B. Dupas, S. Degorge, T. Rodallec, L. Laroche, V. Borderie, and C. Baudouin.** 2007. In vivo confocal microscopy in fungal keratitis. *Br. J. Ophthalmol.* **91:**588–591.
5. **Butler, L. M., S. Khan, G. E. Rainger, and G. B. Nash.** 2008. Effects of endothelial basement membrane on neutrophil adhesion and migration. *Cell. Immunol.* **251:**56–61.
6. **Chang, C. C., S. M. Lieberman, and P. V. Moghe.** 1999. Leukocyte spreading behavior on vascular biomaterial surfaces: consequences of chemoattractant stimulation. *Biomaterials* **20:**273–281.
7. **Cormack, B. P., G. Bertram, M. Egerton, N. A. Gow, S. Falkow, and A. J. Brown.** 1997. Yeast-enhanced green fluorescent protein (yEGFP): a reporter of gene expression in *Candida albicans*. *Microbiology* **143**(Pt. 2)**:**303–311.
8. **Cummings, R. J., S. Mitra, E. M. Lord, and T. H. Foster.** 2008. Antibody-labeled fluorescence imaging of dendritic cell populations in vivo. *J. Biomed. Opt.* **13:**044041.
9. **Doyle, T. C., K. A. Nawotka, C. B. Kawahara, K. P. Francis, and P. R. Contag.** 2006. Visualizing fungal infections in living mice using bioluminescent pathogenic *Candida albicans* strains transformed with the firefly luciferase gene. *Microb. Pathog.* **40:**82–90.
10. **Enjalbert, B., A. Rachini, G. Vediyappan, D. Pietrella, R. Spaccapelo, A. Vecchiarelli, A. J. Brown, and C. d'Enfert.** 2009. A multifunctional, synthetic *Gaussia princeps* luciferase reporter for live imaging of *Candida albicans* infections. *Infect. Immun.* **77:**4847–4858.
11. **Faust, N., F. Varas, L. M. Kelly, S. Heck, and T. Graf.** 2000. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood* **96:**719–726.
12. **Filipe-Santos, O., P. Pescher, B. Breart, C. Lippuner, T. Aebischer, N. Glaichenhaus, G. F. Spath, and P. Bousso.** 2009. A dynamic map of antigen recognition by CD4 T cells at the site of *Leishmania major* infection. *Cell Host Microbe* **6:**23–33.
13. **Gerami-Nejad, M., K. Dulmage, and J. Berman.** 2009. Additional cassettes for epitope and fluorescent fusion proteins in *Candida albicans*. *Yeast* **26:**399–406.
14. **Hernandez, R., and S. Rupp.** 2009. Human epithelial model systems for the study of *Candida* infections in vitro. Part II. Histologic methods for studying fungal invasion. *Methods Mol. Biol.* **470:**105–123.
15. **Holmes, H., J. C. Kennedy, R. Pottier, R. Rossi, and G. Weagle.** 1995. A recipe for the preparation of a rodent food that eliminates chlorophyll-based tissue fluorescence. *J. Photochem. Photobiol. B* **29:**199.
16. **Keppler-Ross, S., C. Noffz, and N. Dean.** 2008. A new purple fluorescent color marker for genetic studies in *Saccharomyces cerevisiae* and *Candida albicans*. *Genetics* **179:** 705–710.
17. **Ledbetter, E. C., N. L. Irby, and S. G. Kim.** 2011. In vivo confocal microscopy of equine fungal keratitis. *Vet. Ophthalmol.* **14:**1–9.
18. **MacCallum, D. M., and F. C. Odds.** 2005. Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. *Mycoses* **48:**151–161.
19. **Mitra, S., K. Dolan, T. H. Foster, and M. Wellington.** 2010. Imaging morphogenesis of *Candida albicans* during infection in a live animal. *J. Biomed. Opt.* **15:**010504.
20. **Naglik, J. R., P. L. Fidel, Jr., and F. C. Odds.** 2008. Animal models of mucosal *Candida* infection. *FEMS Microbiol. Lett.* **283:**129–139.
21. **Peters, N. C., J. G. Egen, N. Secundino, A. Debrabant, N. Kimblin, S. Kamhawi, P. Lawyer, M. P. Fay, R. N. Germain, and D. Sacks.** 2008. In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies. *Science* **321:**970–974.
22. **Pietrella, D., A. Rachini, A. Torosantucci, P. Chiani, A. J. Brown, F. Bistoni, P. Costantino, P. Mosci, C. d'Enfert, R. Rappuoli, A. Cassone, and A. Vecchiarelli.** 2010. A beta-glucan-conjugate vaccine and anti-beta-glucan antibodies are effective against murine vaginal candidiasis as assessed by a novel in vivo imaging technique. *Vaccine* **28:**1717–1725.
23. **Saville, S. P., A. L. Lazzell, A. K. Chaturvedi, C. Monteagudo, and J. L. Lopez-Ribot.** 2008. Use of a genetically engineered strain to evaluate the pathogenic potential of yeast cell and filamentous forms during *Candida albicans* systemic infection in immunodeficient mice. *Infect. Immun.* **76:**97–102.
24. **Schweizer, A. and K. Schröppel.** 2009. Experimental infection of rodent mammals for fungal virulence testing. *Methods Mol. Biol.* **470:**141–149.
25. **Sohn, K., and S. Rupp.** 2009. Human epithelial model systems for the study of *Candida* infections in vitro. Part I. Adhesion to epithelial models. *Methods Mol. Biol.* **470:** 95–104.
26. **Spellberg, B., A. S. Ibrahim, J. E. Edwards, Jr., and S. G. Filler.** 2005. Mice with disseminated candidiasis die of progressive sepsis. *J. Infect. Dis.* **192:**336–343.



COLOR PLATE 6 (CHAPTER 33) *C. albicans* 24 h after injection. GFP-expressing *C. albicans* strain YAW3 was injected into a DBA/2NCr mouse approximately 24 h before imaging. Images were taken at a depth of ~50 µm in a mouse ear in vivo. Images were taken using the Olympus FV1000 microscope at the University of Rochester Confocal and Conventional Microscopy core, with 488-nm excitation and a 20×, 0.75-NA objective. The image presented is a projection of a 15-image *z*-stack (1 µm/slice). 10.1128/9781555817176.ch33cp6



COLOR PLATE 7 (CHAPTER 33) *C. albicans* yeasts form filaments and attract phagoyctes within 4 h after injection. mCherry-expressing *C. albicans* strain SKY38 was injected into a LysM-GFP-expressing mouse approximately 4 h before imaging. Images were taken using the Olympus FV1000 microscope at the University of Rochester Confocal and Conventional Microscopy core, with 488-nm and 559-nm excitation and a 20×, 0.75-NA objective. The image presented is a projection of a 9-image *z*-stack (1.87 µm/slice). 10.1128/9781555817176.ch33cp7

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10.1128/9781555817176.ch34

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Cool Tools 5: The *Candida albicans* ORFeome Project

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Over recent years, there has been a significant increase in the number of available fungal genome sequences. This trend is likely to become more pronounced in the near future with the development of next-generation sequencing technologies, which have rendered genome sequencing cheaper and faster than ever. Although annotated genome sequences are now available, the characterization of each fungal gene remains a challenge. While the predicted function of some genes could be assigned based on sequence homology only, the majority of fungal genes remain uncharacterized. The growing availability of whole-genome data sets has encouraged a shift towards the development of functional genomics and systems biology, allowing analysis of high-throughput whole-genome assays to better understand biological networks. This chapter presents the strategy we have developed to generate a *Candida albicans* ORFeome collection in a versatile Gateway vector, which allows the transfer of the cloned genes into a variety of *C. albicans* Gateway-compatible expression vectors.

The *C. albicans* genome sequencing effort was initiated in October 1996 by the Stanford Genome Technology Center and resulted in the release of a refined assembly 21 in 2007 (16, 34). The genome annotation project has identified ca. 6,200 open reading frames (ORFs), among which 4,700 (75.78%) are still uncharacterized. The classical method to elucidate gene function is by constructing null mutants and testing for phenotypes caused by loss-of-function mutations. In fungi, and particularly the human pathogen *C. albicans*, which is constitutively diploid and lacks a full sexual cycle, overexpression-based screens are a powerful alternative to loss-of-function approaches, which can often be problematic and time-consuming. In other organisms, numerous studies showed that phenotypes produced by gene overexpression may provide important clues to gene function (31, 32). The overexpression of one protein can interfere with the formation of an active stoichiometric complex or can increase the activity of a normal cellular process, which might lead to a detectable phenotype. An overexpression strategy has been used, for example, in the model yeast *Saccharomyces cerevisiae* and has successfully led to the discovery of new signaling pathways (31) as well as new functions and target genes for transcription factors (3). Interestingly, overexpression approaches allowed identification of new cell cycle genes that were not identified in loss-of-function screens in *S. cerevisiae* (32). An advantage conferred by the overexpression strategy is the possibility to study the function of essential genes, for which null mutants cannot be obtained. This strategy is also particularly valuable in the analysis of the function of genes that are members of multigene families. Classical gene deletion of one member of a gene family may not confer a demonstrable phenotype due to functional redundancy within the family or compensatory activation of other family members. However, overexpression of one family member may result in altered cellular properties. The construction of an overexpression collection also enables suppressor screens to be performed to determine whether overexpression of certain genes can complement the genetic or chemical blockade of specific pathways. Despite the advantages associated with an overexpression approach, one should keep in mind that overexpression libraries have their own caveats. While it is known that overexpression can result in impaired growth and identify genes that are involved in cell cycle regulation (32), it cannot be excluded that overexpression-dependent toxicity is not a direct consequence of a protein function. Growth defects associated with toxicity could bias the results of drug screens performed on overexpression collections.

The development of collections of overexpression strains is facilitated by the preestablishment of a complete set of cloned ORFs, or ORFeome. Importantly, ORFeomes represent useful resources for the implementation of other approaches used to elucidate gene function apart from gene deletion or overexpression. Additional approaches include those aimed at evaluating protein subcellular localization/abundance (15) and identifying protein-protein interactions (yeast two hybrid) both at steady state and in response to environmental stimuli (11). Large-scale cloning projects, with the goal of cloning all predicted ORFs into flexible recombinational vectors, have been described for several organisms, including *Caenorhabditis elegans* (27), *Brucella melitensis* (8), *Plasmodium falciparum* (1), *S. cerevisiae* (12),

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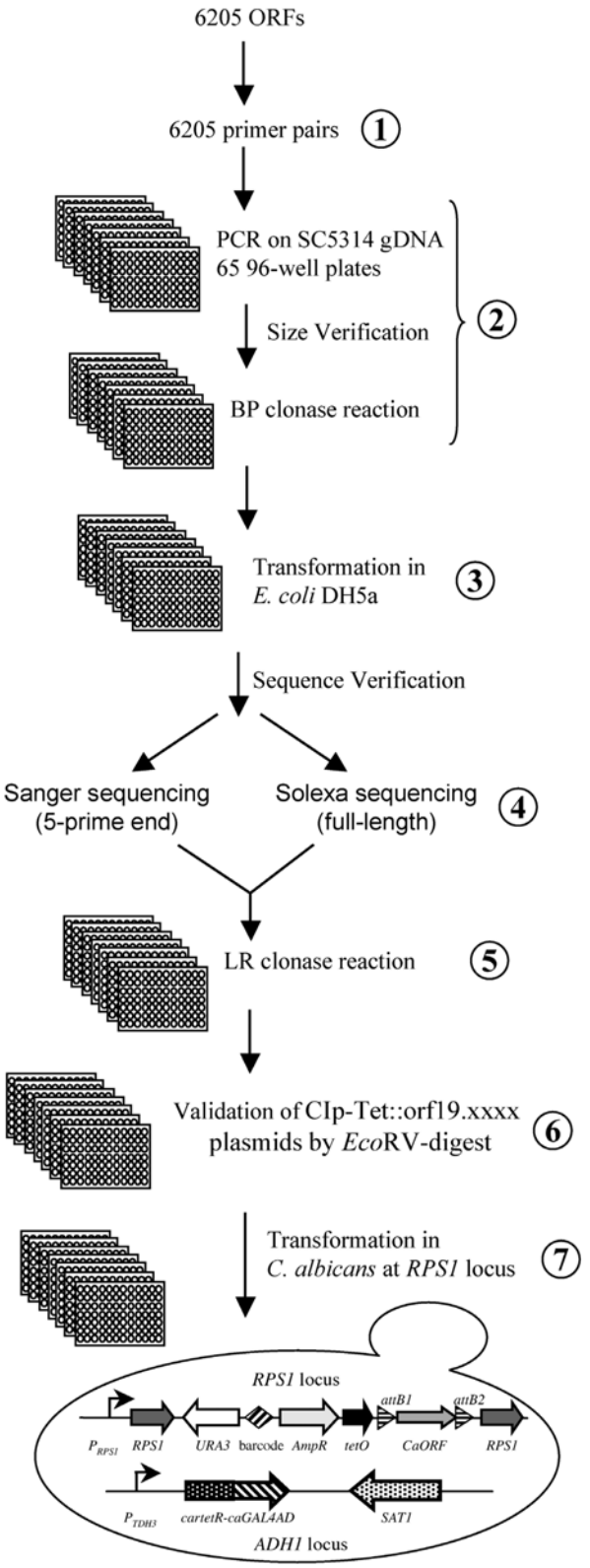

Step 1: Pairs of primers specific for *C. albicans* predicted ORFs are designed. 5-prime primers include the ORF start codon while 3-prime primers do not include the stop codon. Primers are synthesized by groups of 96 with ORFs of increasing size, plate 1 including the smallest ORFs and plate 65 including the largest ORFs.

Step 2: The ORF are amplified from genomic DNA of *C. albicans* strain SC5314 using high fidelity polymerase. PCR products are gel verified for size and quantity and subsequently transferred into the pDONR207 donor vector using BP clonase.

Step 3: BP clonase reactions are transformed into *E. coli* and one transformant per ORF is stored as well as the remainder of the transformation mixture. This will allow the future identification of additional pDONR plasmids for each ORF corresponding to the second allele of the ORF.

Step 4: pDONR plasmids are individually prepared and subjected to Sanger sequencing from the 5-prime end of the ORF in order to ensure proper clone allocation in 96-well plates. Furthermore, the pDONR plasmids are subjected to Solexa sequencing and the resulting sequencing data are compared to ORF sequences obtained from CGD and SNP data generated by Solexa sequencing of *C. albicans* strain SC5314. These data are used to decide whether to proceed to Step 5 or reiterate the procedure, *eg* if mutations in the cloned ORF are not validated from Solexa sequencing data from *C. albicans* strain SC5314.

Step 5: ORFs in pDONR plasmids are individually transferred into a barcoded destination vector CIp10-TETp-GTW, using LR clonase, resulting in the CIp-Tet::orf19.xxxx plasmid.

Step 6: CIp-Tet::orf19.xxxx plasmids are individually prepared and gel verified following a *Eco*RV-digest.

Step 7: *Stu*I or *I-Sce*I-digested CIp-Tet::orf19.xxxx plasmids are transformed in a *C. albicans* strain constitutively expressing a *C. albicans*-adapted reverse Tet-dependent transactivator, allowing doxycycline-inducible expression of the ORF in the CIp-Tet::orf19.xxxx plasmid. Integration of the CIp-Tet::orf19.xxxx plasmid at the *RPS1* locus is verified by colony PCR.

Schizosaccharomyces pombe (19), viruses (25), and *Escherichia coli* K-12 (26). The first human ORFeome has been generated and made publicly available (17). These ORFeome collections are essential to bridge the gap between genome annotation and systems biology and allow large-scale protein characterization. Different techniques exist to generate ORFeomes, but some are more suitable for high-throughput or mass cloning. The *S. cerevisiae* ORFeome was constructed by gap repair cloning (14), a method for cloning that uses yeast homologous recombination. A practical limitation to gap repair cloning is that the clones are locked into the configuration of the original vector. A recombinational cloning approach, using the Gateway cloning system from Invitrogen, was used to construct the ORFeome of the worm *C. elegans*, with a cDNA library as a template (27), and has become the method of choice for the establishment of ORFeomes. Indeed, this system for mass cloning provides compatibility between resource collections, not only for a single organism but also across collections from different organisms.

Individual characterization of mutants on the genome-wide scale can be slow and laborious. In this respect, signature-tagged mutagenesis (STM) provides an attractive alternative. In STM, each mutant is tagged with a different DNA sequence, allowing all tags to be amplified from the DNA of mixed populations of mutants in a single PCR (20). The tag sequence acts as a strain identifier of each mutant in mixed populations. DNA signature tags (or molecular barcodes) facilitate functional screens by identifying mutants in mixed populations that have a reduced or increased adaptation to a particular environment. Several methods have been used for tag detection, including barcode microarrays and next-generation DNA sequencing technologies (Bar-seq) (30). One of the advantages of Bar-seq, apart from its increased robustness compared to microarrays, is the possibility to design multiplex experiments. STM screens have uncovered numerous biological insights regarding microorganisms. For instance, STM of *Salmonella enterica* serovar Typhimurium led to the discovery of a specialized type 3 secretion system (29). In *Mycobacterium tuberculosis*, two separate STM studies demonstrated that a complex cell wall lipid, phthiocerol dimycocerosate, is necessary for survival of the bacterium in the lungs (2, 4). Finally, a variety of STM screens have been conducted using *S. cerevisiae* heterozygous diploid knockout mutants to identify the extent of haploinsufficiency, the state in which the loss of one of two alleles leads to a phenotype (7). In addition, barcoded haploid knockout mutants have been used to identify genes whose inactivation confers increased sensitivity or resistance to drugs or synthetic lethality when combined with another gene knockout (5, 13). Several collections of barcoded heterozygous and homozygous insertion or deletion mutants have been engineered to allow STM screens in *C. albicans*: the GRACE (gene replacement and conditional expression) collection (28), the CaFT (*Candida albicans* fitness test) collection (36), a tagged transposon mutant collection (23), and a collection of tagged deletion mutants obtained by targeted gene replacements (22). Yet, only a fraction of the *C. albicans* genes has been included in these resources (1,152 genes for GRACE, 2,868 for CaFT, 4,239 genes for the tagged transposon mutant collection described by Oh et al. [23], and 674 genes for the knockout mutant collection described by Noble et al. [22]) and a freely available genome-wide collection of *C. albicans* barcoded deletion mutants is still lacking.

The main aim of the *Candida albicans* ORFeome project (Fig. 1) is to develop a genome-wide collection of *C. albicans* barcoded overexpression strains suitable for STM screens in vitro and in vivo. To this end, we have initiated the cloning of the *C. albicans* ORFeome using the versatile Gateway system (35), which allows its transfer into many different expression vectors. The Gateway technology employs the recombination system of bacteriophage lambda with two sets of reactions: the BP reaction, catalyzed by Gateway BP clonase, facilitates recombination between *att*B and *att*P sequences, and the LR reaction, catalyzed by Gateway LR clonase, promotes recombination between *att*L and *att*R sequences. The high-throughput methods we have adopted that take advantage of the highly efficient Gateway technology to first generate a *C. albicans* ORFeome collection and to shuttle the cloned genes into vectors for overexpression in *C. albicans* are described in detail elsewhere (V. Cabral, M. Chauvel, A. Firon, M. Legrand, A. Nesseir, S. Bachellier-Bassi, Y. Chaudhary, C. Munro, and C, d'Enfert, in press). In the first step, the ORFs are amplified by PCR and cloned into a pDONR vector. In the *C. albicans* ORFeome, the start codon of the 6,205 ORFs (sizes ranging from 90 to 15,114 bp) has been included, whereas the stop codon has been excluded to allow insertion of C-terminal tags. We favored C-terminal tagging since tags at the amino termini may possibly interfere with targeting of proteins to the secretory pathway. PCR-amplified ORFs were precipitated and inserted into the Gateway-compatible vector pDONR207 by site-specific recombination. Gateway reactions were performed in 96-well plates, even if the PCR products were not visible after gel electrophoresis, as we found that small amounts of PCR fragments, sometimes undetectable by ethidium bromide staining, can be sufficient for a successful BP reaction. *E. coli* DH5α was then transformed with the BP products in a 96-well plate format. The resulting bacterial transformants were selected on agar containing gentamicin. One clone for each transformation reaction was selected for plasmid preparation. To ensure that they contain the expected ORF and that no mutations were generated during the PCR step, we sequenced (i) the 5′ end of each cloned ORF using standard Sanger sequencing and (ii) pools of plasmids using the Solexa/Illumina technology. This way, appropriate assignment of plasmids in 96-well plates is confirmed and ORFs are fully sequence verified. Plasmids can also be verified by digestion with BsrGI, which cuts on each side of the cloned ORF. This first version of the *C. albicans* ORFeome (version 1.1) will provide the coding sequences in a user-friendly format amenable to high-throughput functional genomics and proteomics experiments.

In the second step, the entry clone collection is transferred to destination vectors allowing tetracycline-dependent expression of *C. albicans* ORFs (Fig. 2A). These destination vectors are derivatives of the CIp10 integrative plasmid (21), which is easily targeted to the *C. albicans RPS1* locus. The vectors allow Gateway-mediated cloning of ORFs downstream of a promoter whose activation is mediated by

FIGURE 1 Workflow of the *C. albicans* ORFeome project. Shown is the overall scheme for high-throughput recombinational cloning of the *C. albicans* ORFs using the Gateway system. Quality control is monitored and documented using PCR and DNA sequencing. The main steps are briefly summarized on the right in the order they are carried out. 10.1128/9781555817176.ch34f1

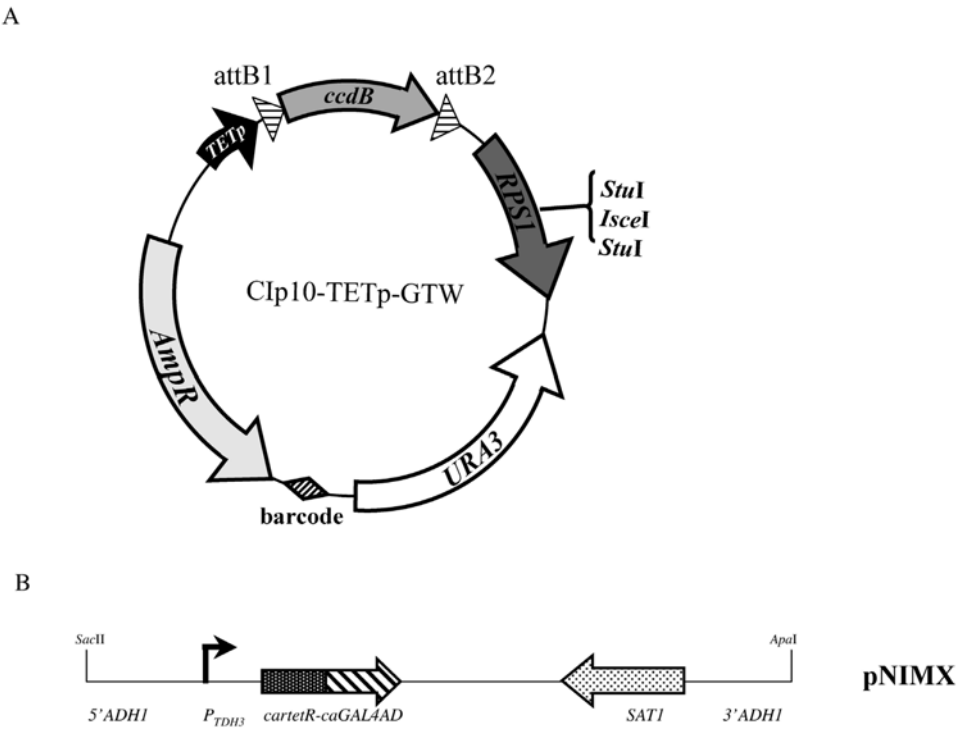

FIGURE 2 Plasmids used in this work. (A) Schematic map of the CIp10-TETp-GTW destination vector used for overexpression of *Candida albicans* ORFs. ORFs cloned in a Gateway donor vector can be transferred through Gateway-mediated recombination at *attR1* and *attR2* of CIp10-TETp-GTW overexpression vectors. The *URA3* gene is used for selection of *C. albicans* transformants. Derivatives of these overexpression vectors can be targeted to the *C. albicans RPS1* locus when linearized with StuI or I-SceI. Expression of the cloned ORFs is achieved when *C. albicans* cells are grown in the presence of doxycycline, which binds to the tetracycline-dependent transactivator rtTA, allowing transcriptional activation at the Tetp promoter. Each expression plasmid is tagged with a unique 12-bp barcode, enabling growth phenotypes of individual strains to be analyzed in parallel. (B) Structure of the tetracycline-dependent transactivator cassette contained in plasmid pNIMX. Unique restriction sites to excise the entire cassette are indicated. P_{TDH3}, basal promoter of *CaTDH3*; *SAT1*, nourseothricin resistance gene; *cartetR-CaGAL4AD*, *Candida albicans*-adapted reverse tetracycline-dependent transactivator *cartTA* gene. 10.1128/9781555817176.ch34f2

the tetracycline-dependent transactivator (rtTA) when tetracycline is added (24) and are individually marked with unique 12-bp tags. This subset of barcoded overexpression plasmids will be used to transform a *C. albicans* strain previously modified to carry pNIMX, a derivative of pNIM1 (24). pNIM1 is a plasmid that carries the rtTA-coding gene under the control of the *C. albicans ADH1* promoter and a green fluorescent protein (GFP) reporter gene under the control of an rtTA-dependent promoter (24). In order to increase the level of expression of ORFs placed under the control of the rtTA-dependent promoter on CIp10 derivatives, we have constructed pNIMX, in which the *C. albicans ADH1* promoter has been replaced with the *C. albicans TDH3* promoter, allowing increased rtTA levels, and the GFP reporter gene of pNIM1 has been deleted, avoiding a dilution effect of rtTA (Fig. 2B). pNIMX has been tested with the gLUC59 reporter gene (10), and it was found that the expression levels were 30 times higher than those obtained with pNIM1 (S. Bachellier-Bassi, M. Legrand and C. d'Enfert, unpublished data). Preliminary results obtained in our laboratories using subcollections of overexpression mutants constructed using the strategy outlined above showed that these can be successfully used individually or in an STM screen to identify genes whose overexpression interferes with antifungal sensitivity (S. Znaidi and C. d'Enfert, unpublished data) or genome integrity (M. Legrand, R. Loll, and C. d'Enfert, unpublished data).

The construction of the *C. albicans* ORFeome constitutes a novel and important step towards a systems biology analysis of *C. albicans*. Its availability will allow the implementation of new postgenomics approaches to better understand the biology of *C. albicans*. For instance, an ORFeome collection will encourage large-scale interactome mapping projects. Because of an unusual codon usage of *C. albicans*, *S. cerevisiae* is not the ideal host for yeast two-hybrid experiments with *C. albicans* proteins. A new two-hybrid system with *C. albicans* itself as a host has been recently developed (33). The *C. albicans* ORFeome library can be transferred into the prey plasmid, thereby reducing the number of transformants that would be necessary to cover the entire interactome network if a genomic library in the prey plasmid was used. In addition, the ORFeome will allow biochemical genomics approaches to assay each *C. albicans* protein for a biochemical activity of interest (18). The ORFeome will

also make possible *C. albicans* proteome chips (37). To facilitate the study of the *C. albicans* proteome, the proteins could be overexpressed, purified, and then printed onto slides to form a *C. albicans* proteome microarray and screened for the ability to interact with proteins, phospholipids, or sera. The arrays can also be used for substrate identification of a kinase of interest (6), in order to develop a kinase-substrate map for *C. albicans*, to screen for protein-drug interaction, and to detect posttranslational modifications. As mentioned above, the development of a collection that can be used in STM screens will facilitate the investigation of numerous aspects of *C. albicans* biology. Additional libraries of strains expressing tagged proteins (e.g., GFP and tandem affinity purification [TAP] tag) could also be constructed to define the cellular location of proteins or posttranslational modifications such as phosphorylation and glycosylation. Overall, a panel of such libraries should facilitate the systematic execution of high-throughput biochemical and microscopic assays of the *C. albicans* proteome. Finally, the construction of the ORFeome could pave the way to the generation of the *C. albicans* promoterome (library of gene promoters) in order to better characterize expression profiles (9). Building on these collections (and the methods described in this paper), we hope that the *C. albicans* community will expand both the quantity and type of proteome level data available.

Work in the laboratories of C.D. and C.M. is supported by the Wellcome Trust (The Candida albicans ORFeome project, WT088858MA). Work in the laboratory of C.D. is also supported by the Agence Nationale de la Recherche (KANJI, ANR-08-MIE-033-01) and the European Commission (FINSysB, PITN-GA-2008-214004).

REFERENCES

1. **Aguiar, J. C., J. LaBaer, P. L. Blair, V. Y. Shamailova, M. Koundinya, J. A. Russell, F. Huang, W. Mar, R. M. Anthony, A. Witney, S. R. Caruana, L. Brizuela, J. B. Sacci, Jr., S. L. Hoffman, and D. J. Carucci.** 2004. High-throughput generation of *P. falciparum* functional molecules by recombinational cloning. *Genome Res.* **14:**2076–2082.
2. **Camacho, L. R., D. Ensergueix, E. Perez, B. Gicquel, and C. Guilhot.** 1999. Identification of a virulence gene cluster of *Mycobacterium tuberculosis* by signature-tagged transposon mutagenesis. *Mol. Microbiol.* **34:**257–267.
3. **Chua, G., Q. D. Morris, R. Sopko, M. D. Robinson, O. Ryan, E. T. Chan, B. J. Frey, B. J. Andrews, C. Boone, and T. R. Hughes.** 2006. Identifying transcription factor functions and targets by phenotypic activation. *Proc. Natl. Acad. Sci. USA* **103:**12045–12050.
4. **Cox, J. S., B. Chen, M. McNeil, and W. R. Jacobs, Jr.** 1999. Complex lipid determines tissue-specific replication of *Mycobacterium tuberculosis* in mice. *Nature* **402:**79–83.
5. **Decourty, L., C. Saveanu, K. Zemam, F. Hantraye, E. Frachon, J. C. Rousselle, M. Fromont-Racine, and A. Jacquier.** 2008. Linking functionally related genes by sensitive and quantitative characterization of genetic interaction profiles. *Proc. Natl. Acad. Sci. USA* **105:**5821–5826.
6. **Dephoure, N., R. W. Howson, J. D. Blethrow, K. M. Shokat, and E. K. O'Shea.** 2005. Combining chemical genetics and proteomics to identify protein kinase substrates. *Proc. Natl. Acad. Sci. USA* **102:**17940–17945.
7. **Deutschbauer, A. M., D. F. Jaramillo, M. Proctor, J. Kumm, M. E. Hillenmeyer, R. W. Davis, C. Nislow, and G. Giaever.** 2005. Mechanisms of haploinsufficiency revealed by genome-wide profiling in yeast. *Genetics* **169:** 1915–1925.
8. **Dricot, A., J. F. Rual, P. Lamesch, N. Bertin, D. Dupuy, T. Hao, C. Lambert, R. Hallez, J. M. Delroisse, J. Vandenhaute, I. Lopez-Goni, I. Moriyon, J. M. Garcia-Lobo, F. J. Sangari, A. P. Macmillan, S. J. Cutler, A. M. Whatmore, S. Bozak, R. Sequerra, L. Doucette-Stamm, M. Vidal, D. E. Hill, J. J. Letesson, and X. De Bolle.** 2004. Generation of the *Brucella melitensis* ORFeome version 1.1. *Genome Res.* **14:**2201–2206.
9. **Dupuy, D., Q. R. Li, B. Deplancke, M. Boxem, T. Hao, P. Lamesch, R. Sequerra, S. Bosak, L. Doucette-Stamm, I. A. Hope, D. E. Hill, A. J. Walhout, and M. Vidal.** 2004. A first version of the *Caenorhabditis elegans* Promoterome. *Genome Res.* **14:**2169–2175.
10. **Enjalbert, B., A. Rachini, G. Vediyappan, D. Pietrella, R. Spaccapelo, A. Vecchiarelli, A. J. Brown, and C. d'Enfert.** 2009. A multifunctional, synthetic *Gaussia princeps* luciferase reporter for live imaging of *Candida albicans* infections. *Infect. Immun.* **77:**4847–4858.
11. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. *Nature* **340:**245–246.
12. **Gelperin, D. M., M. A. White, M. L. Wilkinson, Y. Kon, L. A. Kung, K. J. Wise, N. Lopez-Hoyo, L. Jiang, S. Piccirillo, H. Yu, M. Gerstein, M. E. Dumont, E. M. Phizicky, M. Snyder, and E. J. Grayhack.** 2005. Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Dev.* **19:**2816–2826.
13. **Hillenmeyer, M. E., E. Fung, J. Wildenhain, S. E. Pierce, S. Hoon, W. Lee, M. Proctor, R. P. St. Onge, M. Tyers, D. Koller, R. B. Altman, R. W. Davis, C. Nislow, and G. Giaever.** 2008. The chemical genomic portrait of yeast: uncovering a phenotype for all genes. *Science* **320:** 362–365.
14. **Hudson, J. R., Jr., E. P. Dawson, K. L. Rushing, C. H. Jackson, D. Lockshon, D. Conover, C. Lanciault, J. R. Harris, S. J. Simmons, R. Rothstein, and S. Fields.** 1997. The complete set of predicted genes from *Saccharomyces cerevisiae* in a readily usable form. *Genome Res.* **7:** 1169–1173.
15. **Huh, W. K., J. V. Falvo, L. C. Gerke, A. S. Carroll, R. W. Howson, J. S. Weissman, and E. K. O'Shea.** 2003. Global analysis of protein localization in budding yeast. *Nature* **425:**686–691.
16. **Jones, T., N. A. Federspiel, H. Chibana, J. Dungan, S. Kalman, B. B. Magee, G. Newport, Y. R. Thorstenson, N. Agabian, P. T. Magee, R. W. Davis, and S. Scherer.** 2004. The diploid genome sequence of *Candida albicans*. *Proc. Natl. Acad. Sci. USA* **101:**7329–7334.
17. **Lamesch, P., N. Li, S. Milstein, C. Fan, T. Hao, G. Szabo, Z. Hu, K. Venkatesan, G. Bethel, P. Martin, J. Rogers, S. Lawlor, S. McLaren, A. Dricot, H. Borick, M. E. Cusick, J. Vandenhaute, I. Dunham, D. E. Hill, and M. Vidal.** 2007. hORFeome v3.1: a resource of human open reading frames representing over 10,000 human genes. *Genomics* **89:**307–315.
18. **Martzen, M. R., S. M. McCraith, S. L. Spinelli, F. M. Torres, S. Fields, E. J. Grayhack, and E. M. Phizicky.** 1999. A biochemical genomics approach for identifying genes by the activity of their products. *Science* **286:** 1153–1155.
19. **Matsuyama, A., R. Arai, Y. Yashiroda, A. Shirai, A. Kamata, S. Sekido, Y. Kobayashi, A. Hashimoto, M. Hamamoto, Y. Hiraoka, S. Horinouchi, and M. Yoshida.** 2006. ORFeome cloning and global analysis of protein localization in the fission yeast *Schizosaccharomyces pombe*. *Nat. Biotechnol.* **24:**841–847.
20. **Mazurkiewicz, P., C. M. Tang, C. Boone, and D. W. Holden.** 2006. Signature-tagged mutagenesis: barcoding mutants for genome-wide screens. *Nat. Rev. Genet.* **7:**929–939.

21. **Murad, A. M., P. R. Lee, I. D. Broadbent, C. J. Barelle, and A. J. Brown.** 2000. CIp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* **16:**325–327.
22. **Noble, S. M., S. French, L. A. Kohn, V. Chen, and A. D. Johnson.** 2010. Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. *Nat. Genet.* **42:**590–598.
23. **Oh, J., E. Fung, U. Schlecht, R. W. Davis, G. Giaever, R. P. St. Onge, A. Deutschbauer, and C. Nislow.** 2010. Gene annotation and drug target discovery in *Candida albicans* with a tagged transposon mutant collection. *PLoS Pathog.* **6:**e1001140.
24. **Park, Y. N., and J. Morschhauser.** 2005. Tetracycline-inducible gene expression and gene deletion in *Candida albicans*. *Eukaryot. Cell* **4:**1328–1342.
25. **Pellet, J., L. Tafforeau, M. Lucas-Hourani, V. Navratil, L. Meyniel, G. Achaz, A. Guironnet-Paquet, A. Aublin-Gex, G. Caignard, P. Cassonnet, A. Chaboud, T. Chantier, A. Deloire, C. Demeret, M. Le Breton, G. Neveu, L. Jacotot, P. Vaglio, S. Delmotte, C. Gautier, C. Combet, G. Deleage, M. Favre, F. Tangy, Y. Jacob, P. Andre, V. Lotteau, C. Rabourdin-Combe, and P. O. Vidalain.** 2010. ViralORFeome: an integrated database to generate a versatile collection of viral ORFs. *Nucleic Acids Res.* **38:**D371–D378.
26. **Rajagopala, S. V., N. Yamamoto, A. E. Zweifel, T. Nakamichi, H. K. Huang, J. D. Mendez-Rios, J. Franca-Koh, M. P. Boorgula, K. Fujita, K. Suzuki, J. C. Hu, B. L. Wanner, H. Mori, and P. Uetz.** 2010. The *Escherichia coli* K-12 ORFeome: a resource for comparative molecular microbiology. *BMC Genomics* **11:**470.
27. **Reboul, J., P. Vaglio, J. F. Rual, P. Lamesch, M. Martinez, C. M. Armstrong, S. Li, L. Jacotot, N. Bertin, R. Janky, T. Moore, J. R. Hudson, Jr., J. L. Hartley, M. A. Brasch, J. Vandenhaute, S. Boulton, G. A. Endress, S. Jenna, E. Chevet, V. Papasotiropoulos, P. P. Tolias, J. Ptacek, M. Snyder, R. Huang, M. R. Chance, H. Lee, L. Doucette-Stamm, D. E. Hill, and M. Vidal.** 2003. *C. elegans* ORFeome version 1.1: experimental verification of the genome annotation and resource for proteome-scale protein expression. *Nat. Genet.* **34:**35–41.
28. **Roemer, T., B. Jiang, J. Davison, T. Ketela, K. Veillette, A. Breton, F. Tandia, A. Linteau, S. Sillaots, C. Marta, N. Martel, S. Veronneau, S. Lemieux, S. Kauffman, J. Becker, R. Storms, C. Boone, and H. Bussey.** 2003. Large-scale essential gene identification in *Candida albicans* and applications to antifungal drug discovery. *Mol. Microbiol.* **50:**167–181.
29. **Shea, J. E., M. Hensel, C. Gleeson, and D. W. Holden.** 1996. Identification of a virulence locus encoding a second type III secretion system in *Salmonella typhimurium*. *Proc. Natl. Acad. Sci. USA* **93:**2593–2597.
30. **Smith, A. M., L. E. Heisler, J. Mellor, F. Kaper, M. J. Thompson, M. Chee, F. P. Roth, G. Giaever, and C. Nislow.** 2009. Quantitative phenotyping via deep barcode sequencing. *Genome Res.* **19:**1836–1842.
31. **Sopko, R., D. Huang, N. Preston, G. Chua, B. Papp, K. Kafadar, M. Snyder, S. G. Oliver, M. Cyert, T. R. Hughes, C. Boone, and B. Andrews.** 2006. Mapping pathways and phenotypes by systematic gene overexpression. *Mol. Cell* **21:**319–330.
32. **Stevenson, L. F., B. K. Kennedy, and E. Harlow.** 2001. A large-scale overexpression screen in *Saccharomyces cerevisiae* identifies previously uncharacterized cell cycle genes. *Proc. Natl. Acad. Sci. USA* **98:**3946–3951.
33. **Stynen, B., P. Van Dijck, and H. Tournu.** 2010. A CUG codon adapted two-hybrid system for the pathogenic fungus *Candida albicans*. *Nucleic Acids Res.* **38:**e184.
34. **van het Hoog, M., T. J. Rast, M. Martchenko, S. Grindle, D. Dignard, H. Hogues, C. Cuomo, M. Berriman, S. Scherer, B. B. Magee, M. Whiteway, H. Chibana, A. Nantel, and P. T. Magee.** 2007. Assembly of the *Candida albicans* genome into sixteen supercontigs aligned on the eight chromosomes. *Genome Biol.* **8:**R52.
35. **Walhout, A. J., G. F. Temple, M. A. Brasch, J. L. Hartley, M. A. Lorson, S. van den Heuvel, and M. Vidal.** 2000. GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Methods Enzymol.* **328:**575–592.
36. **Xu, D., B. Jiang, T. Ketela, S. Lemieux, K. Veillette, N. Martel, J. Davison, S. Sillaots, S. Trosok, C. Bachewich, H. Bussey, P. Youngman, and T. Roemer.** 2007. Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans*. *PLoS Pathog.* **3:**e92.
37. **Zhu, H., M. Bilgin, R. Bangham, D. Hall, A. Casamayor, P. Bertone, N. Lan, R. Jansen, S. Bidlingmaier, T. Houfek, T. Mitchell, P. Miller, R. A. Dean, M. Gerstein, and M. Snyder.** 2001. Global analysis of protein activities using proteome chips. *Science* **293:**2101–2105.

10.1128/9781555817176.backm

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