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Metarhizium spp., Cosmopolitan Insect-Pathogenic Fungi: Mycological Aspects

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I. Introduction

Fungi of the hyphomycete genus *Metarhizium* have been isolated from infected insects and soil of all continents except Antarctica, and there have been isolations from near the Antarctic Circle (Roddam and Rath, 1997). Although some isolates of these fungi have rather restricted host

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ranges, the group is better known for its ability to kill a wide spectrum of insects, including insects in at least seven orders (Veen, 1968). The common name for *Metarhizium*-induced disease is ''green muscardine,'' based on the encrustation of insect cadavers with green conidia.

Metarhizium anisopliae was the first fungus worldwide to be mass produced and utilized for insect-pest control (Krassilstschik, 1888; Steinhaus, 1975). There have been many attempts, with varying levels of success, in the ensuing more than 100 years to use *Metarhizium* spp. for pest control. *Metarhizium* spp. historically are second only to *Beauveria* spp. as the fungi most frequently used in the field against insects; there is some indication that the roles of these two fungi are, or soon will be, reversed. This genus also has been the subject of molecular- level studies on fundamental topics such as virulence and host specificity; currently it probably is the best understood entomopathogenic fungal genus at these basic levels. Mycological aspects of *Metarhizium* spp. will be reviewed here, and interactions of these fungi with their hosts (including biocontrol of arthropod-pests) will be published next year.

The current worldwide research effort on *Metarhizium* spp. is intense. A *Web of Science* search of literature by the keyword *Metarhizium* from 1988–2001 provided about 800 hits, and this does not include reports in the proceedings of many scientific meetings, nor many book chapters. Also, active research groups in non-English-speaking countries (especially in eastern Asia) are significantly underrepresented. From 12 research papers in 1998, the number tripled by 1992, tripled again by 1997, and output has remained stable through 2001 at approximately 85 papers per year. The rapid increase in research on *Metarhizium*, followed by sustained high scientific output, can be explained by several important worldwide attitude changes and the initiation of several promising *Metarhizium*-based pest-control and molecular-biology efforts.

- First, the previously expressed, but seldom acted on, interest in reducing the environmental chemical-pesticide load started receiving serious governmental attention almost worldwide. The Food and Agriculture Organization (FAO) of the United Nations, in recognition of the short and long-term hazards of chemical pesticides, especially to developing nations, has recently recommended reducing pesticide-environmental load by encouraging developing and using integrated pest management (IPM) programs. IPM, as defined by FAO (2002), includes encouraging natural pest control mechanisms.

- Organic farming became an established and growing part of agriculture. Although currently small (probably 3% in the U.S.) it is a large enough segment to be financially attractive to niche marketers such as producers of biological control agents. The definitions and required procedures for organic agriculture were recently codified in the United States. In that synthetic chemicals are banned, these regulations encourage microbial control.
- The devastating plagues of locusts in Africa in the mid-1980s—and the widespread recognition that grasshoppers and minor plagues of several locusts are persistent and on going problems throughout Africa, Australia, South America, United States, and other places—inspired several agencies in North America and Northern Europe to fund efforts to be prepared with something besides chemical pesticides in the event of another serious plague cycle and to control perennial grasshopper problems. Among the groups so charged was a combined unit of CAB International in England and the International Institute for Tropical Agriculture (IITA) in Cotonou, Benin, which identified itself under the acronym LUBILOSA (Lutte Biologique contre les Locustes et Sauteriaux = Biological Control of Locusts and Grasshoppers) and quickly expanded to include as collaborators or sponsors many agencies and scientists interested in locust control. After screening a number of fungi, LUBILOSA decided to emphasize an isolate of *M. anisopliae* var. *acridum* from an African grasshopper. (This isolate was originally identified as *M. flavoviride*.) The banning of Dieldrin, the previous first line of defense in locust and grasshopper control—and problems associated with the organophosphates which replaced it—provided an unprecedented level of urgency to the insect-fungus research. LUBILOSA was well funded for 12 years (14 million U.S. dollars for the first 10 years). Since the work centered almost totally on *Metarhizium*, it served to advance substantially the knowledge on this group of fungi. Groups in Australia and Brazil also provided new *Metarhizium* isolates and new basic and applied information on these fungi. Commercial products for locust and grasshopper control have been developed in both Africa and Australia.
- LUBILOSA and others have obtained high insect mortality in the field at low (~30%) relative humidity following ultra-low volume (ULV) application with conidia formulated in oil. This observation counters previous thinking that fungal infection will occur only at very high RH and thereby greatly increases possible hosts and host environments for *Metarhizium* and other entomopathogenic fungi.

- Responses by insects, particularly grasshoppers and locusts, to infection by fungus—including *Metarhizium*—which delay disease development has engendered considerable research activity. Of primary interest has been ''behavioral fever''—that is, basking in sunlight to raise hemolymph temperature above the optimum for the fungal parasite.
- The control of scarabiid beetle larvae with *Metarhizium* in sugarcane and pastures has provided much information, and commercial products are now available in Australia.
- Spittlebugs are serious pests in Central and South America, where major projects are underway to develop *Metarhizium* to protect sugarcane and pasture. An estimated 500,000 ha. were treated with *Metarhizium* in Brazil in 2001 for spittlebug control.
- Molecular techniques were used to solve taxonomic problems within the genus *Metarhizium*, to conduct ecological studies, to transform isolates with foreign DNA, and to identify virulence factors.
- *Metarhizium* research was aided by advances with other fungi, not the least of which was *B. bassiana*. For the first time, through the efforts of MycoTech (now Emerald AgriScience), a spore product with wide host range was readily available. This relieved insect pathologists of producing fungus for their field experiments, and provided them a level of standardization never before possible. As might be expected, there has been some cross feeding between the findings of the *Beauveria* research and projects based on *Metarhizium*.
- Finally, the potential for medical use of *Metarhizium* metabolites was proven, which resulted in a large number of studies on destruxins and swainsonine.

The most important taxonomic reviews of *Metarhizium* are the revisions by Tulloch (1976), Rombach *et al.* (1986d), and Driver *et al.* (2000). To our knowledge, there has been no major review of general biological and molecular biology studies on the single genus *Metarhizium*. There are, however, a number of reviews and books that include general information and methods for working with entomopathogenic fungi including *Metarhizium* spp. The reader is directed to Roberts and Hajek, 1992; Lomer and Prior, 1992; Tanada and Kaya, 1993; Goettel and Johnson, 1997; Lacey, 1997; Pu and Li, 1996 (in Chinese); Hajek, 1997; Alves, 1998 (in Portuguese); Boucias and Pendland, 1998; Bridge *et al.*, 1999; Burges, 1998a; Hall and Menn, 1999; Lacey and Kaya, 2000; Gurr and Wratten, 2000; Butt *et al.*, 2001a; and Khetan, 2001.

II. Nomenclature

The first known species of the genus *Metarhizium* Sorokin was one isolated from infected insects in the Ukraine in the late 1870s. It was originally named *Entomophthora anisopliae* based on the generic name of its scarab host, *Anisoplia austriaca* (Metschnikoff, 1879)[1] and renamed *Isaria destructor* the next year (Metschnikoff, 1880). Both generic placements were incorrect, so a new generic name, *Metarhizium* (spelled with one ''r''), was proposed for this fungus by Sorokin (1883). The original specific name ''*anisopliae*'' was retained. Later, Pettit (1895) used double ''r'' (*Metarrhizium*) in discussing the identification by Roland Thaxter, a U.S.A. expert on insect-associated fungi, of similar fungi found in New York. The genus was reviewed in 1976 by Margaret Tulloch. At that time, the genus was universally spelled with a double ''r.'' Although Veen (1968) recommended the extra ''r'' be retained, Tulloch (1976) proposed that the second ''r'' be dropped and the spelling returned to that of the original description. A confusing factor in this issue is that the International Code of Botanical Nomenclature (Greuter *et al.*, 2000) specifies that the original spelling be retained except for the correction of ortho- or typographic errors. There is an orthographic error in the single ''r'' version. The name is a compound name (meta + rhizium); as pointed out by Stearn (1992), when the initial r (rho) of a Greek word is transliterated as ''rh,'' an additional ''r'' should be added to it—if in forming a compound word it will be preceded by a vowel. Stearn also points out that this rule has not been well recognized (including by Linnaeus), and selection of the one or two ''r'' form is optional. He recommends the author's original spelling be accepted. The insect pathology and taxonomic literature since 1976 has almost universally adhered to the single ''r'' spelling, and in our opinion this should be continued. This essentially is the *de facto* situation. A *Current Contents* search of the recent (January 1998–September 2002) literature showed 17 entries with the

[1]Metschnikoff's publication (Metschnikoff, 1879) of the name *E. anisopliae* was in Russian. He used three transliterations of his name through his life, ending with ''Metchnikoff'' (note the deleted ''s'') during his long tenure at Pasteur Institute. Nevertheless, he used ''Metschnikoff'' in 1880 (Metschnikoff, 1880) in a publication in German. This is the form used today almost universally by fungal taxonomists—for example, Tulloch (1976); Carmichael *et al.* (1980); Humber and Hansen (2001); and, in our opinion, it is the one that should be retained for *M. anisopliae* (in contrast to Zimmermann *et al.*, 1995), since it is the transliteration he selected within one year of his describing the first *Metarhizium* species. Also, a fellow Russian, Sorokin (1883), while proposing the genus *Metarhizium*, used the ''Metschnikoff'' transliteration in quoting the 1879 Metschnikoff paper.

double ''r'' spelling—11 of which used both ''r'' and ''rr''—and 401 single ''r'' *Metarhizium* papers. Most of the ''rr'' papers are on chemical (metabolite) or mammalian-health studies.

III. Taxonomy

Several species of *Metarhizium* were described prior to 1976, but Tulloch (1976) accepted only *Metarhizium anisopliae* and *M. flavoviride*; all other species were synonymized or treated as varieties. She divided the *M. anisopliae* into short-spore isolates (ranging up to 8 μm in length), which she called variety *anisopliae*, and long-spore isolates (10 μm up to 14 μm) which she referred to as variety *major* (now referred to as *majus* [Rombach *et al.*, 1986d]). The separation of species strictly on morphology and sometimes color proved unsatisfactory for the wide number of isolates held in fungus collections worldwide since 1976. For example, the isozyme study of St. Leger *et al.* (1992a) indicated there were a number of cryptic varieties or species in the ARS Entomopathogenic Fungus Collection in Ithaca, New York. There have been efforts to add a molecular level to the taxonomic studies, and identification methods based on physiology and/or nucleic acids have been attempted in recent years (e.g., Bidochka *et al.*, 1994, 2001b; Bridge *et al.*, 1993, 1997; Cobb and Clarkson, 1993; Curran *et al.*, 1994; Fegan *et al.*, 1993; Fungaro *et al.*, 1996; Hegedus and Khachatourians, 1993; Inglis *et al.*, 1999; Leal *et al.*, 1994b, 1997; Mavridou and Typas, 1998; Nam *et al.*, 1998; Pantou, 2003; Pereira Jr. *et al.*, 1995; Rakotonirainy *et al.*, 1994; Tigano-Milani *et al.*, 1995; Yoon *et al.*, 1999). The most expansive of recent examinations of the comparative taxonomic status of *Metarhizium* isolates is that of Driver *et al.* (2000). This study included 123 fungal isolates from many locations and several fungus collections worldwide but included no Eastern European, Chinese or North American isolates. Their DNA-based methods included RAPD-PCR (random amplified polymorphic DNA) and sequence data from the ITS, 5.8S rDNA, and 28S rDNA D3 regions. A partition homogeneity test indicated no incongruence in phylogenetic signal amongst the ITS1, 5.8S rDNA, ITS2, and D3 expansion regions of 28S rDNA data. RAPD-PCR groups correlated fairly well with ITS-sequence identity groups. The taxonomic questions which informed this study were presented, along with a summary of the data and their interpretations, by Driver and Milner (1995). The outcome of the study was to propose that the genus *Metarhizium* be divided into 10 clades. (''Clade'' means ''branch,'' and signifies a phylogenetic group or lineage of organisms.) Three specific names *M. album, M. flavoviride*,

and *M. anisopliae* were retained. *M. flavoviride* was separated into 5 clades and *M. anisopliae* into 4 clades. These were assigned variety names rather than subspecies names. One physiological trait, viz., the ability to grow at temperatures below 10 °C (Rath *et al.*, 1995), was considered not taxonomically meaningful. Insufficient data caused one clade to be assigned a letter rather than variety name, viz. *M. flavoviride* Type E. The clades, their names and abbreviations used in this review are given in Table I. Driver *et al.* (2000) list the diagnostic ITS1 and ITS2 sequences for the ''type'' isolate for each clade. They conclude that their data supports (a) the monophyly of *M. anisopliae* (clades 7–10); (b) the monophyly of the core group of *M. flavoviride* (clades 4–6); (c) the monophyly of clade 7, the locust/grasshopper (acridoid) *M. anisopliae* var. *acridum* group which previously was considered primarily *M. flavoviride*; and (d) a sister group relationship between the acridoid group (clade 7) and *M. anisopliae* (clades 8–10).

The clades described by Driver *et al.* (2000) are intended to represent ''evolutionary significant groups'' or ''biologically meaningful clusters,'' and they can be expected to serve as a solid base on which to construct future developments in *Metarhizium* taxonomy. The clades tend to confirm several older morphologically or biologically based studies. For example, the proposal of *M. flavoviride* var. *minus* (Rombach *et al.*, 1986d) and reinstatement of *M. album* (Rombach *et al.*, 1987) were supported and accepted. Useful changes include recognition that a rather homogeneous group of acridoid isolates (clade 7) was clearly associating with *M. anisopliae* rather than *M. flavoviride* and placing them in a separate variety, *M. anisopliae* var. *acridum*. On the other hand, a number of Australasian isolates previously considered to be *M. anisopliae* were recognized as *M. flavoviride* var. *novazealandicum* (clade 3) or *M. flavoviride* var. *flavoviride* (clade 6). The Driver *et al.* (2000) clade separations were basically validated in a recent extensive *Metarhizium* IGS sequence, group-I intron, and complete ribosomal DNA study (Pantou *et al.*, 2003). For example, 39 of 40 isolates of *Ma-an* were placed in one clade; and other varieties were recognized as separate clades.

The numerous molecular taxonomy studies on *Metarhizium* spp. conducted over the past 10 years have mostly excluded North American isolates. Nevertheless, the catalog of the ARSEF Collection in Ithaca, New York, indicates that it is very likely that *Ma-an* is the predominant variety in this part of the world. The RAPD/PCR study of Cobb and Clarkson (1993) and Bidochka *et al.* (1994) clustered together the type (IMI 168777 [Tulloch, 1976]) of *Ma-an* from Eritrea with ARSEF 2575 (=Me1 = IIBC 191–613) from the U.S.A. There is no

TABLE I

DIVISION OF *METARHIZIUM* SPP. ISOLATES INTO CLADES BASED PRIMARILY ON DNA CHARACTERISTICS (ITS AND rDNA SEQUENCES, AS WELL AS RAPD-PCR GROUPS) (DRIVER *ET AL.*, 2000)

Clade	Species	Variety (abreviation)	Previous names	Hosts groups	Location
1	*M. album*		*M. album*	Homoptera	Philippines
2	*M. flavoviride*	var. Type E	*M. f.* var. *flavoviride*	Homoptera	Brazil
			M. a. var. *anisopliae*	Coleoptera	Australia
3	*M. flavoviride*	var. *novazealandicum (Mf-no)*	*M.a.* var. *anisopliae* *M.a.* var. *frigidum*	Lepidoptera	New Zealand
			M.a. var. *novazealandicum*	Coleoptera soil	Australia
4	*M. flavoviride*	var. *pemphigum (Mf-pe)*	*M.a.* var. *anisopliae*	Homoptera	United Kingdom
5	*M. flavoviride*	var. *minus (Mf-mi)*	*M.f.* var. *minus*	Homoptera	Philippines
6	*M. flavoviride*	var. *flavoviride (Mf-fl)*	*M.a.* var. *frigidum*	Coleoptera	France Germany
			M.f. var. *flavoviride*	Isoptera mound	Australia
7	*M. anisopliae*	var. *acridum (Ma-ac)*	*M.f.* var. *minus*	Orthoptera	Africa Australia
			M.a. var. *anisopliae*	Brazil Mexico	
8	*M. anisopliae*	var. *lepidiotum (Ma-le)*	*M.a.* var. *anisopliae*	Coleoptera Isoptera	Australia
9	*M. anisopliae*	var. *anisopliae (Ma-an)*	*M.a.* var. *anisopliae*	Orthoptera, Isoptera, Coleoptera, Diptera, Hemiptera	Benin, Eritrea, Oman, Pakistan, Thailand, Australia, New Zealand, Mexico, Brazil, USA*
10	*M. anisopliae*	var. *majus (Ma-ma)*	*M.a.* var. *majus*	Coleoptera	Philippines Indonesia

*Presence of *M.a.* var. *anisopliae* in USA is based on ARSEF culture collection catalogue (Humber and Hansen, 2001).

evidence at present that other varieties of *Ma-an* nor the other two species (*M. album* and *M. flavoviride*) are present in this area. A DNA-level survey of North American isolates, such as that by Driver *et al.* (2000) is needed. An allozyme study of endonuclease digestion analysis (RFLP) of mtDNA separated 25 isolates of *Ma-an* into 20 groups, and this method has been proposed for characterizing strains (Mavridou and Typas, 1998). Also, it should be noted that 120 *Metarhizium* spp. isolates proved separable into 48 classes by allozyme analysis based on eight enzyme activities, suggesting the existence of several ''cryptic'' species (St. Leger *et al.*, 1992b). A search for 28S rDNA group I introns in 40 *Ma-an* isolates revealed their presence in 5 isolates (Mavridou *et al.*, 2000; Neuveglise *et al.*, 1997). All five introns were at identical positions with those from other organisms belonging to various phyla.

The ITS sequences of *Ma-majus* are more closely related to *Ma-an* than to any of the other varieties. Nevertheless, DNA-based studies to date have placed *Ma-majus* isolates in a clearly separate group, (e.g., Driver *et al.*, 2000; Yoon *et al.*, 1999). Because the size (volume) of these spores is approximately double that of most *Ma-an* isolates, it is possible that they may be stable diploids. In fact, some of the *majus* isolates studied by DNA restriction fragment length polymorphisms (RFLPs) were heterozygous diploids (Pipe *et al.*, 1995); an isozyme study of several isolates indicated that, with the exception of a laboratory-forced homozygous diploid, all other *Ma-ma* examined were heterozygous at one to four loci (St. Leger *et al.*, 1992b). It is interesting that the variety *Ma-ma* is frequently isolated from scarabiid larvae, which suggests that, compared to other insect groups, double infection with compatible isolates may be more likely to produce stable diploids in scarabs than in other insects. With double infection of a chrysomelid beetle with two isolates of *Ma-an*, only one or the other isolate was detected in 52 of 53 dead hosts. Both genotypes were detected in the 53rd host insect, and one single-spore isolate from that had a novel band by RAPD analysis. Although the mechanism is unknown, transfer of repetitive DNA, rather than diploidization and the parasexual cycle, is suggested since this would explain new bands rather than just the segregation of parental bands (Leal-Bertioli *et al.*, 2000). Heterokaryons of *B. bassiana* produced in caterpillar hemolymph were not stable and reverted to parent strain characteristics (Wang *et al.*, 2002).

Sexual (perfect or teliomorph) stages of *Metarhizium* spp. are extremely rare. An early report by Schaerffenberg (1959) of a Sphaeriales ascospore stage for *M. anisopliae* has been ignored as probably incorrect

(Tulloch, 1976). The imperfect (anamorph) fungus cultured from the *Cordyceps taii* perfect stage in 1988 in the People's Republic of China was proposed as a new species of *Metarhizium, M. taii* (Liang *et al.*, 1991). This is now synonymized under a previously described species, *M. quizhouensis* (Liu *et al.*, 2001). Unfortunately, cultures of this fungus were not included in the Driver *et al.* (2000) study. Judging from the appearance of this *Metarhizium* and BLAST comparisons of its ITS sequences, it probably is *M. anisopliae* var. *anisopliae*. Two of its distinguishing features, the occasional septate conidium and synnemata production *in vitro*, have been seen in other *M. anisopliae* var. *anisopliae* cultures (Ribeiro *et al.*, 1992) and probably are not viable species designators (Humber, personal communication). *Cordyceps brittlebankisoides* also was reported to have a *Metarhizium* anamorph (imperfect stage), which was identified as *M. anisopliae* var. *majus* (Liu *et al.*, 2001, 2002). *Cordyceps* anamorphs initially identified as *M. biformisporae* and *M. cylindrosporae* were later transferred to *Nomuraea* (Liu *et al.*, 2001; Tzean *et al.*, 1993). *Metarhizium pinshaensis* also was described from the PRC (Guo *et al.*, 1986). The ITS, 5.8S and 28S rDNA regions of the very common caterpillar pathogen *Nomuraea rileyi* suggest it is more closely related to *Metarhizium* than it is to other *Nomuraea* spp. (Boucias *et al.*, 2000). Interestingly, rDNA studies of yeast-like endosymbiotes (YLSs) of planthoppers (Homoptera: Delphacidae) establish that they are in fact not true yeasts but are related to filamentous *Ascomycetes*, including *Cordyceps* and its imperfect stages such as *Metarhizium* and *Beauveria* (Suh *et al.*, 2001).

The perfect stage of *Metarhizium* does not seem to have ever been produced *in vitro*. Nevertheless, immature *C. taii* stroma on field-collected dead insects were matured by Liang *et al.* (1991) by burying the cadavers with their heads exposed in soil under a bell jar. The jar was removed and replaced at 48-hr. intervals for ventilation. The soil was kept moist and the temperature approximately 20 °C. Some *Metarhizium* isolates make asexual synnemata in culture that somewhat resemble *Cordyceps* structures (e.g., Rombach *et al.*, 1986d).

Although homothallism is possible, known perfect stages of *Metarhizium* suggest mating types of the fungus. Discovery of teleomorphs of *Metarhizium* should be more frequently collected than they have been if homothallic matings were both common and productive. Driver *et al.* (2000) and Bidochka *et al.* (2000) attempted to find mating types of *Metarhizium* by using *nit* mutants with impaired nitrogen metabolism. All strains that gave positive results in vegetative-compatibility-group (VCG) complementation tests had closely related genotypes. There were, however, minor variations in the RAPD profiles of these

strains (Driver *et al.*, 2000). There is no mention of producing perfect stages of *Metarhizium* in these attempts. Accordingly, the biological species concept of breeding populations to delimit taxa is not directly applicable to *Metarhizium* spp. The fungus is known to have a parasexual cycle with an unstable diploid stage (Al-Aidroos, 1980; Messias and Azevedo, 1980; Valadares-Ingles and Azevedo, 1997), as well as parameiosis in which recombinants are obtained directly from heterokaryons without a diploid phase (Bagagli *et al.*, 1991), but these phenomena have not been reported in the field. St. Leger *et al.* (1992a) detected the existence of a clonal population of *Metarhizium* which ranged throughout Brazil into Colombia. Processes such as vegetative or nuclear incompatibility may serve to genetically isolate strains. The need for teleomorphs (perfect stages) of imperfect fungi with large populations, such as *Metarhizium*, was recently questioned and proposed as probably not necessary for species survival (Bidochka and De Koning, 2001).

Comparisons of published taxonomic studies on *Metarhizium* spp. are sometimes difficult because almost all fungal collections and many laboratories assign new numerical designations to all isolates as they are accessioned, regardless of whether the isolates are newly discovered or from another culture collection. The result is that the same isolate may be studied by several research groups, but the reader may think different isolates have been studied if each study reports its findings for that isolate under a different number. In some cases, a report will list one of the ''synonym'' accession numbers, which is of great assistance. If the original host, date of collection, and site of collection are listed, it is sometimes possible to recognize the isolate and group the new data with other information on that isolate. We suggest that authors give, in addition to their own accession numbers, the original designation (and others known to the writer) for each isolate. This could take the form of a column in a table listing all isolates used in a particular study or a web address leading the reader to this information.

IV. Double-Stranded RNA Viruses (Mycoviruses)

Double-stranded RNA viruses have been widely reported in filamentous fungi. Such viruses can severely reduce virulence of plant pathogens, particularly the fungus *Cryphonectria parasitica*, which causes chestnut blight (Chen *et al.*, 1996; Choi and Nuss, 1992; Wang and Nuss, 1995). Reduction in virulence and/or conidiogenesis would be a serious problem in the use of *Metarhizium* spp. for insect control. Beginning in the mid 1990s, double-stranded RNAs (dsRNA) have been

reported in several isolates of *Ma-an* and *Ma-ac* worldwide, and mycovirus particles with diameters of about 35 nm have been purified and partially characterized. The dsRNAs in purified particles correspond quite well to those detected in mycelium, and the viruses have been assigned to the families *Totiviridae* and *Partitiviridae* (Gimenez-Pecci *et al.*, 2002). They are not present in some isolates widely used for research and for field, applications, e.g. *Ma-an* ARSEF #2575 (=Me1) (Melzer and Bidochka, 1998) and ARSEF #925 (=E9) (Bogo *et al.*, 1996) as well as *Ma-ac* isolate IMI330189 (the Green Muscle isolate of LUBILOSA) (Martins *et al.*, 1999). On the other hand, the highly virulent and UV tolerant *Ma-ac* isolate ARSEF #324 does contain double-stranded dsRNA mycoviruses (Martin *et al.*, 1999; Melzer and Bidochka, 1998). A comparison of *Ma-an* isolates free of dsRNA and with dsRNA suggested that the dsRNA-free isolates were slightly more virulent to ticks than their counterparts; but, since there were other differences in these isolates, this conclusion is tentative (Frazzon *et al.*, 2000). In general, efforts to ''cure'' isolates of dsRNA have met with little or no success. Nevertheless, curing was obtained in one of 100 isolates by virus exclusion through conidiogenesis by Melzer and Bidochka (1998), and in comparison with the parent with dsRNA, the dsRNA-free variant proved more virulent and had increased growth and conidiospore production. A similar result was reported by Gimenez-Pecci *et al.* (2002), in that an isolate with spontaneous loss of some high-molecular-weight dsRNA had significant alterations in colony morphology and increased conidiogenesis. On the other hand, two clones of a *Ma-an* culture which had been stored in very different ways were found to be with and without dsRNA, but there was no difference in virulence of these isolates to aphids (Leal *et al.*, 1994a). Forcing heterokaryons composed of dsRNA-free and dsRNA-containing *Ma-an* Brazilian isolates did not result in transfer of the mycovirus(s) (Bogo *et al.*, 1996), while forced heterokaryosis between an African *Ma-ac* isolate without dsRNA and an Australian *Ma-ac* isolate with dsRNA succeeded in transfering the dsRNA to the African isolate. There appeared to not be significant loss in virulence through the acquiring of the dsRNA by the African isolate (Martins *et al.*, 1999). dsRNA bands have been proposed as characters for identifying *Metarhizium* isolates. Bidochka *et al.* (2000), however, found that bands of similar size from different isolates may differ by Northern analysis, which rules out dsRNA for isolate identification. This study also found that multiple dsRNA infections can occur in a single *Ma-an* isolate, and dsRNAs generally are harbored in genetically related fungal strains. In summary, there is some indication of deleterious effects

on *Metarhizium* spp. by mycoviruses, but to date, total loss of virulence (hypovirulence) has not been associated with the presence of dsRNA nor dsRNA viruses.

V. Toxins

Toxins produced by *Metarhizium in vitro* and potentially present in biocontrol formulations and/or in fungus-killed insects were examined as to safety to nontarget organisms (Strasser *et al.*, 2000), with the conclusion that *Ma* will not secrete copious metabolites into the environment and that the toxins do not pose a health risk. Several actions to assure safety—for example, developing less complicated monitoring systems, were suggested.

A. Destruxins

The destruxins (Dxs) are composed of an alpha-hydroxy acid and five amino acid residues joined by amide and ester linkages to form cyclic structures. There are 38 Dxs or Dx analogs (Pedras *et al.*, 2002), which is double the 19 known in 1989 (Gupta *et al.*, 1989). They are divided chemically into five basic groups labeled A through E, plus several subgroups of each. Almost all are reported from various isolates of *Ma-an*, with Dx A, B, and E tending to predominate in culture, and based on host symptoms and extraction of moribund insects, some Dxs are present in biologically significant (toxic) levels before insect death. Not all isolates of *Ma-an* produce destruxins (e.g., ARSEF #1080), and *Ma-ac* and *Ma-ma* are poor Dx producers. It is probably significant that specialist strains produce little or no toxins. They have evolved a pathogenic relationship that involves growing within the living host. Specialization is therefore associated with a ''growth strategy'' and opportunism with a ''toxin strategy.'' This variation in Dx production is illustrated in a comparison of 4 *Ma*, 3 *Ma-ma*, 1 *M. album*, and 2 *M. flavoviride* (probably *Ma-ac*) isolates (Amiri-Besheli *et al.*, 2000). The *in vitro* production of Dx A, B, and E was highest for one of the *Ma-an* isolates (ARSEF 23), and the lowest also was a *Ma-an* (V220 = ARSEF 441). Production was very low in the non-*Ma-an* isolates. Interestingly, when insects were infected with 7 of the above 10 isolates, all of which produced Dx E in artificial medium, extracts of cadavers universally failed to contain detectable amounts of Dx E, whereas both Dx A and Dx B were produced by 6 and 3 of the isolates, respectively. Two *Ma-an* isolates [ARSEF 23 and 2575 (=Me1)], which differed approximately 10 fold in *in vitro* crude Dx production were similar *in vivo*.

The first two Dxs were isolated in Japan by Kodaira (1961, 1962). In the 1960s, the Japanese called *Ma-an* ''*Oospora destructor*,'' a name proposed by Delacroix (1893) but quickly abandoned in the western world. Based on the name *O. destructor*, the toxins were named ''destruxins.'' Although the great majority of Dxs were isolated from *Metarhizium*, Dx B also has been reported from the plant pathogen *Alternaria brassicae*, both *in vitro* and *in vivo* (Ayer and Pena-Rodriguez, 1987; Bains and Tewari, 1987; Pedras and Smith, 1997), while Dx A4 and A5 (toxic to *Drosophila*) and homodestruxin B (not toxic to *Drosophila*) were isolated from an entomopathogenic *Aschersonia* sp. culture (Krasnoff *et al.*, 1996). Also, a Dx B analog recently was isolated from *Beauveria felina* (Kim *et al.*, 2002). The newest group is pseudodestruxin A and B produced by the coprophilous fungus *Nigrosabulum globosum* (Che *et al.*, 2001). Natural Dx analogs include bursaphelocides A and B, roseocardin and roseotoxin B, all from hyphomycetous fungi. They were not named Dxs because they were first isolated from fungal species different from *M. anisopliae* (Pedras *et al.*, 2002).

The destruxins have been heavily researched by insect pathologists, plant pathologists and microbiologists, particularly because of their usefulness in understanding disease. They have also received considerable attention from biochemists and natural-products chemists because of their very interesting cyclic hexadepsipeptide structure and the question of their synthesis *in vitro* and *in vivo* by fungi. Chemical synthesis methods are described in detail by Pedras *et al.* (2002). The biosynthetic pathway is assumed to be through a non-ribosomal multifunctional enzyme system (Kleinkauf and von Dohren, 1987, 1996; Peeters *et al.*, 1990; Turner, 2000). This biosynthesic process is used by *B. bassiana* to produce beauvericin (another cyclic depsipeptide) (Peeters *et al.*, 1988). For most fungi, the multifunctional enzyme is encoded by a single gene, *pes* (Weber *et al.*, 1994). Peptide synthetase (PES)-encoding genes (*pes*) all exhibit similar subunit structure that includes a separate domain responsible for the activation and polymerization of each residue. Six conserved regions, termed *core sequences*, have been identified within each domain (Haese *et al.*, 1993; Marahiel, 1992). Highly conserved oligonucleotide sequences from the core 1 and core 2 regions of known *pes* genes were used to screen (probe) a genomic library of *Ma-an* strain Me1 (=ARSEF #2575), which is a known destruxin producer (Bailey *et al.*, 1996). A clone containing 14.5 kb of genomic DNA was selected and sequenced. The amino acid sequence inferred from the nucleotide sequence of this clone showed conserved domain sequences similar to the core motif known for other multifunctional PESs. This study found, for the first time, introns within

the *pes* gene. There are no reports of attempts to transform non-Dx-producing isolates with this large gene, but the sequence information may prove useful in constructing non-Dx-producing *Ma* strains.

An effort to optimize Dx production resulted in ''modest high levels'' of 49 and 268 mg L^{-1} for Dx A and Dx B, respectively, in liquid media (Liu *et al.*, 2000); and 3 mg kg^{-1} and 227 mg kg^{-1} on semisolid medium at 71% water content (Liu and Tzeng, 1999). Addition of a cyclopeptolide (#90-215) increased Dx production up to 12-fold in liquid fermentation (Espada and Dreyfuss, 1997). Seven single-spore isolates from *Ma-an* spores exposed to mutagens (ethyl methane sulfonate or UV-C) had increases in Dx A, B, and E production of 1.1- to 7.3-fold (Hsiao and Ko, 2001).

The toxicity to insects of Dxs A, B, and E is well documented. Immediate response to injection of Dxs varies with insect group,—for example, larvae of *G. mellonella* (Lepidoptera)—enter into an immediate tetanus of voluntary muscles, whereas Orthoptera do not pass through this phase (Roberts, 1981). Paralysis is probably attributable to muscle depolarization by reversible opening of the gated Ca^{2+} channels in the membrane (Dumas *et al.*, 1996; Samuels *et al.*, 1988). The ionophoric properties of Dx A allow calcium ion mobilization across liposomal membrane barriers (Hinaje *et al.*, 2002). The Dx detoxification process has been examined in *G. mellonella* (Lepidoptera) and *Locusta migratoria* (Orthoptera); see Pedras *et al.* (2002) for details. There are probably several mechanisms involved, but the half-life of Dx in *G. mellonella* is only one hour. This was due to opening of the Dx cyclic structure (Jegorov *et al.*, 1992). Destruxin B is toxic to plants, and its detoxification in the plant has been followed in a series of experiments also summarized in Pedras *et al.* (2002). Aside from its outright toxic affects on insect hosts, destruxins have the more subtle impact of reducing the cellular (encapsulation) immunoresponse of the host—even at doses that do not cause paralysis or any general signs of toxicity (Huxham *et al.*, 1989; Vey *et al.*, 2002).

A study on synthesized Dx A analogs indicated that most analogs had about the same insect toxicity as the parent molecule, Dx A (Ast *et al.*, 2001). The presence of an ester bond apparently is essential for biological activity. Opening the molecule to make it linear rather than cyclic destroys toxicity. The structurally nonrigid protoDx is not toxic, and the most hydrophilic Dxs are the least toxic (Dumas *et al.*, 1994; Suzuki and Tamura, 1972). Toxicity varies with test system, but Dx A and E are generally considered the most cytotoxic of all of the Dxs to insect tissue culture cells and to insects. Tissue culture cell lines from the Colorado potato beetle are sensitive to Dxs and have been suggested

as tools for bioassay and detection of these compounds (Charpentier *et al.*, 2002). The phagodepressant (antifeedant) activity on insects of Dxs apparently causes death by both toxicity and starvation, and exposure to Dxs increased susceptibility to *Ma-an* infection (Amiri *et al.*, 1999). Other biological activities of the Dxs include cytotoxic effects on leukemia cells and spleen lymphocytes, antiproliferative activity on mammalian neoplastic cells in *in vitro* assays with murine neoplasms, suppressive effects on hepatitis B viral surface antigen, induction of erythropoietin, a positive inotropic effect on rat cardiac tissue, reversible inhibition of vacuoloar-type ATPase, and negative effects on viruses in insect tissue culture. Dx E, when used as a vacuolar-type H^+-ATP inhibitor, induced apoptosis in human epidermal carcinoma cells overexpressing EGFR (epidermal growth factor receptor) if the cells were stimulated with EGF (Yoshimoto and Imoto, 2002). Apoptosis was not induced in human tumor cells which do not overexpress EGFR. Thus the toxin may suppress the responses of immunosuppressive hemocytes via the stimulation of key intracellular proteins. These and other biological effects of Dxs are discussed by Pedras *et al.* (2002), Vey *et al.* (2001), and Strasser *et al.* (2000).

A correlation between *in vitro* production of destruxins and fungal virulence to insects has been suggested (Fargues *et al.*, 1985; Kershaw *et al.*, 1999; Vey *et al.*, 2001, 2002). For example, mortality of hosts routinely is several days less for Dx-producing *Ma-an* isolates when compared to non-Dx-producing *Ma-ac* and *Ma-ma* isolates. Nevertheless, as stated by Pedras *et al.* (2002), the role of Dxs in virulence of *Ma* to insects is not clearly evaluated.

B. Swainsonine

Swainsonine, a sugar analogue, is an indolizidine alkaloid molecule with a fused piperidine and pyrrolidine ring system. It was first isolated from the fungal plant pathogen *Rhizoctonia leguminicola*, but its structure was not correctly assigned until it had been rediscovered in an Australian plant, *Swainsona canescens*. It has been extracted from the North American spotted locoweed *Astragalus lentiginosus*. It was reported from *M. anisopliae* isolate F-3622 by Hino *et al.* (1985). This compound is an effective inhibitor of both lysosomal alpha-mannosidase and mannosidase II. The first of these is involved in cellular degradation of polysaccharides and the latter is a key enzyme in the processing of asparagine-linked glycoproteins. Swainsonine has antimetastatic, antitumor-proliferative, and anticancer activity. Swainsonine is the first glycoprotein-processing inhibitor to be

selected for clinical testing as an anticancer drug. It also has immunostimulating activity. Nemr (2000) lists some 24 other mammalian-oriented studies on the biological effects of swainsonine. Its high cost has hindered clinical trials, and *Metarhizium* has been studied as a possible producer of the compound in liquid and solid-substrate fermentation (Patrick *et al.*, 1993, 1995, 1996; Tamerler *et al.*, 1998; Tamerler-Yildir *et al.*, 1997). The highest yield reported was with aerated, modified-starch-casein medium which gave approximately 60 mg swainsonine per liter (Tamerler-Yildir *et al.*, 1997). In addition to fermentation, synthetic methods for producing stereoisomers of swainsonine and its analogues have been investigated (Nemr, 2000). There are no published reports of screening various *Metarhizium* isolates for enhanced swainsonine production. The toxicity of swainsonine to insects needs investigation.

C. Novel Metabolites

Various *Metarhizium* isolates have been reported in the past decade to produce small molecules, which are either toxic to insects or have potential medical usage. For example, *M. flavoviride* from Czechoslovakia was found to produce new compounds, viridoxins A and B, which were toxic to Colorado potato beetle larvae at LC_{50}s of 40 and 50 parts per million, respectively (Gupta *et al.*, 1993). These compounds are closely related to the plant-toxic colletotrichins of *Colletotrichum nicotianae*. Viridoxin A and B differ from the colletotrichins in that the alcohol is esterified. Apparently, *Colletotrichum* lacks the acyl transferase of *Mf*. The difference is very significant biologically. The colletotrichins are toxic to plants and not to insects, whereas the viridoxins are toxic to insects and not to plants. In other words, the two fungi are closely attuned to their native hosts, with one a plant pathogen and the other an insect pathogen. A *Metarhizium* sp. isolated from soil in Japan was found to produce a 12-hydroxy-ovalicin (Kuboki *et al.*, 1999), which was named Mer-f3. It is a small molecule of approximately 311 molecular weight. The parent compound, ovalicin, is known to have immunosuppressive activity. Accordingly, it was examined for mixed lymphocyte culture reaction, and the inhibitory activity of Mer-f3 was similar to that of ovalicin. Mer-f3 also was checked for inhibition of mouse leukemia L-1210 cells, and there was no inhibitory activity against cell growth—which indicates that Mer-f3 may be an immunosuppressent with low mammalian toxicity. There is no report of insect toxicity of this compound. Another *Metarhizium* sp. isolated from soil in Japan was found to produce a

novel immunosuppressive substance, which was named metacytofilin (Iijima *et al.*, 1992), with a molecular weight of about 305. Immunosuppression by this compound was demonstrated in the mixed lymphocyte culture reaction and in delayed-type hypersensitivity. The compound had no cytotoxicity to L-1210 and other carcinoma cells, and it had no antimicrobial activity against bacteria or fungi. A third Japanese *Ma-an* isolate from soil produced three related small molecules: zygosporin D and two new cytochalasins. The purpose of the study was to search for new phytotoxic compounds. Zygosporin D is known to inhibit plant growth, which was confirmed with a seedling assay in this study. The new cytochalasins, however, were of too low toxicity to be considered phytotoxic. One was named deacetylcytochalasin C., and the other was not assigned a trivial name. The role, if any, of these compounds in virulence of *Metarhizium* to insects was not reported (Fujii *et al.*, 2000). Cytochalasins are known to retard cell mobility, and therefore they may interfere with phagocytotsis and encapsulation of the fungal pathogen in insects. Two cytochalasins, C and D, were first isolated from *Ma-an* cultures. A related compound, cytochalasin B, was not toxic to *Galleria mellonella* larvae at 150 μg/g by injection (Roberts, 1981).

In addition to destruxins, swainsonine, and the other small molecules mentioned above, there are larger compounds which have deleterious effects on insects. For example, Mazet *et al.* (1994) found large, heat-labile compounds in *Beauveria*-infected lepidopterous larval blood. This blood was toxic to other caterpillars upon injection. Such studies should be done with *Metarhizium*. Based on the EST and microarray studies described elsewhere in this review, at least one isolate of *Ma-an* has the genetic information to produce a wide range of protein toxins, which are known from organisms ranging from prokaryotes to vertebrates (Freimoser *et al.*, 2002).

VI. Safety

The concept of safety of microbial control agents to non-target organisms has been formalized in many nations. These governments have issued regulations for testing for deleterious effects of microbial control agents on non-target organisms, and new products must be certified as safe before they are allowed into the marketplace. Adherence to these regulations is mandatory in Europe, Australia, and North America. Unfortunately, they sometimes are almost ignored elsewhere. Accordingly, it is indeed fortunate that *Metarhizium* spp. basically are not serious threats to non-target organisms. Quality

control in manufacturing facilities may be more of a threat (Jenkins and Grzywacz, 2000), in that opportunistic fungal pathogens and mycotoxin producers could be present as contaminants in the *Metarhizium* cultures, thereby turning relatively safe products into potentially dangerous ones. In addition to being lethal to its target arthropod pest(s), isolates of *Metarhizium* spp. utilized for pest control must be of minor impact to other (non-target) elements of their environment, particularly beneficial organisms (e.g. honey bees, silkworms, insect predators and parasitoids), other invertebrates, and vertebrates—especially mammals (Goettel *et al.*, 1990b). Adverse reactions can range from allergic responses to infected tissues and death. *Ma-an, Ma-ac, Mf-no* and/or *Mf-fl* have been registered for field use in Australia, Africa, North and South America, and Europe. Stringent nontarget tests were conducted prior to approval of these registrations.

A. Mammals

1. *Mammalian Infection*

The least common among the non-target effects is infection of mammals. The internal temperature of mammals (more than 35 °C) is at or beyond the maximum temperature for growth of most *Metarhizium* spp., with the exception of many *Ma-ac* isolates. Mammalian safety data are part of the packages used to register entomopathogenic fungi with national governments to sell a fungal isolate in that country. Accordingly, these data, which are expensive to obtain, usually are considered proprietary information and sometimes are not immediately or automatically available to the public. A publicly sponsored study with mice, rats, and rabbits that used an *Ma-an* isolate (ARSEF #23) established that deep mycoses did not occur even when conidia were injected intraperitoneally (Shadduck *et al.*, 1982). Granulomas were produced at the sites of injection and fungi could be cultured from organs two weeks after exposure, but not at three weeks. Ocular treatments of rabbits with dry spores and spore suspensions had no serious after effects. Feeding the spores by intubation to rats and heavy dusting of mice with dry conidia also did not produce any symptoms in the animals. Similar rodent studies with LUBILOSA standard *Ma-ac* strain IMI 330189 indicated no infectivity or toxicity and full clearance from the peritoneal cavity and organs (Goettel and Jaronski, 1997). Isolates of *Ma-an* and *Ma-ac* from Madagascar with similar testing did not cause any overt pathogenesis or death; but some animals had enlarged spleens and livers, and multiple white lesions in peritoneal cavities

(summarized by Goettel and Jaronski, 1997). The lesions were assumed to be due to heightened immune responses, and scar tissue was present from encapsulation of spores.

Two cases of human corneal infection (fungal keratitis) have been reported (DeGarcia *et al.*, 1997; Jani *et al.*, 2001). Both were successfully managed with Natamycin 5% (pinaracin) along with other drops and ointments. In the past few years, numerous fungi have been implicated in mycotic keratitis (e.g. *Fusarium, Aspergillus, Acremonium, Penicillium, Candida* and several dematiaceous fungi) (Jani *et al.*, 2001). Mycotic rhinitis has been reported in a cat that had nasal discharge and subcutaneous swelling of the nasal bridge. As in *Aspergillus* and *Penicillium* nasal infections of cats, the *Metarhizium* cultured from this cat penetrated the bone from the nasal cavity to subcutaneous areas. The fungus involved was eventually identified as *Ma-an*. Even though the infection was present for many months prior to treatment, it did not disseminate to other tissues—even with inappropriate dosing of corticosteroides. There was prompt improvement following anti-fungal therapy, but the infection reoccurred three months after discontinuing treatment. The authors propose that the treatment had been terminated prematurely. The cat, however, was euthanized rather than being given a second course of anti-fungal chemicals (Muir *et al.*, 1998). Two cases of sinus infections in humans have been reported from Colombia. In both cases, the fungus-infected tissue was removed by surgery. There was no anti-fungal treatment following surgery and the patients both recovered without complications (Revankar *et al.*, 1999). This study also reported three cases where *Ma-an* was found in bronchoalveolar lavages, but these were considered clinically insignificant. *Aspergillus* is the most common organism implicated in mammalian fungal sinusitis, although dematiaceous fungi such as *Curvularia, Alternaria*, and *Bipolaris* also occur, especially in allergic fungal sinusitis (de Shazo *et al.*, 1997; Morpeth *et al.*, 1996). There are no reported deaths from mammalian infections by *Ma-an*, but an immunocompromised nine-year-old boy who had had acute leukemia for five years and was on chemotherapy developed an invasive *Ma-an* infection in several parts of its body. The child eventually died. The fungus was listed as probably contributing to the death, but was not evaluated as the cause of death (Burgner *et al.*, 1998). In each case where the *Ma-an* cultured from mammalian hosts was tested for anti-fungal drug resistance, drug resistance was detected. In one case the fungus was resistant to several chemicals including Itraconazol, but was sensitive to Amphotericine B (Burgner *et al.*, 1998); and in another case, the isolates were resistant to Amphotericine B but were susceptible to Itraconazol (Revankar *et al.*,

1999). *Metarhizium* is cosmopolitan and almost ubiquitus in soil; nevertheless, the first cases of mammalian infection were not reported until after the mid-1990s. This may be related to recent rapid increases in numbers of immunocompromised individuals due to organ transplants, cancer chemotherapy, steroid treatments, HIV infection, etc. These patients are particularly susceptible to infection by many fungi.

2. *Allergic Reactions*

Allergy to fungi, particularly fungal spores, is a source of hay fever and asthma in humans. The conidia of *Metarhizium* spp. can be expected to cause similar responses in selected individuals. Surprisingly, such reports, at least those backed by solid experimental studies, apparently do not exist in the medical literature. In our experience, opportunities for truly massive exposure to conidia frequently occur in many of the small-scale factories extant worldwide. It is not uncommon for an employee or manager to reach with bare hands into a mass of fungus-covered rice and lift some to demonstrate how nicely the *Metarhizium* has conidiated. One of us (DWR) has seen *Metarhizium*-conidia-collecting areas of small factories in Brazil where the floor was green from large numbers of conidia, or employees had green hair from airborne conidia caused by either huge sieves used to separate conidia from rice or from hammer mills used to mill rice on which conidia were produced. When queried, both management and factory employees stated that allergy problems were neither common nor serious. Nevertheless, the occasional employee does report an allergic response. In one sugar cane cooperative, to protect against developing allergy, the employees were rotated on fifteen-day shifts between growing parasitic insects and producing *Metarhizium*. Those cases of allergy reported were short-term, being gone within 1 to 2 days after terminating exposure. There were no cases of infection by the fungus reported by any of these workers or managers.

Intranasal challenge of mice (10^7 conidia in 50 μl) was safe with all but one Malagasy isolate (Goettel and Jaronski, 1997). The one, a *Ma-an* isolate, induced severe respiratory stress, including some deaths, within minutes of exposure. No lesions were noted at necropsy, and the cause(s) of death remains unexplained. This is a unique event in safety testing of numerous *Metarhizium* isolates worldwide. Halving the dose caused temporary breathing difficulty in the mice but no mortality. Madagascar is well known for exotically different flora and fauna, and it is interesting that the only *Ma-an* isolate to induce lethal responses in a mammal is from this island nation. None of the Malagasy fungi caused eye irritation.

Immune responses in Balb/c mice to *Ma-an* (ARSEF #1080) proteins extracted from mycelium and conidia have been documented (Ward *et al.*, 1998, 2000a,b). Also, conidia were incubated in water with 2% unpurified chitin to encourage production of enzymes, primarily chitinases and proteases. The fungus was removed by filtration, and proteins with molecular weights greater than 3000 were concentrated. Equal protein concentrations of each of the three components were combined to form crude *M. anisopliae* antigen. The antigen was administered to Balb/c mice intraperitoneally with an aluminum hydroxide adjuvant (1.3% alhydrogel). About two weeks after this sensitization the mice were intraperitoneally challenged with the same antigen. The mice exhibited immune and pulmonary inflammatory responses characteristic of allergy. The initial study was extended to look at responses associated with changes in pulmonary response, lung pathology, and cytokine profile in bronchoalveolar-lavage fluid. All treatment groups demonstrated significant nonspecific pulmonary inflammation and pulmonary sensitivity to methacholine one day post intratracheal (IT) challenge, but airway responses returned to normal by three days post exposure. The cytokine profile indicated a Th2-type response, which was reflected in cellular influx and total IgE induction. The conclusion again is that the sensitized mice demonstrated physiological and histopathological changes characteristic of allergic disease. A third study was undertaken to address concerns that the sensitization protocol was artificial and not representative of an environmental exposure and, therefore, could produce allergic responses that would not occur under normal environmental exposure conditions. Accordingly, mice were exposed to the *Metarhizium* mixed soluble antigens without adjuvant and only through respiratory routes. A second group of animals received intraperitoneal (IP) sensitization with the adjuvant followed by intratreacheal (IT) challenge. Both groups exhibited immune and pulmonary responses typical of allergic asthma. In general, local responses in the lung were greater in the IT-sensitized group compared to the IP-sensitized group. IgE levels were greater following IP sensitization; and, therefore, they were considered not the best indicator of allergen respiratory responses. The allergen doses utilized (2.5 to 250 μg) of the mixed antigen is obviously a strong challenge, as was the IT challenge (25 μg). It is unlikely that field use of *Metarhizium* would expose mammals to any of the three antigen sources other than whole conidia, thus making difficult interpretation of these very carefully executed, but laboratory only, studies in relation to field-level fungus use.

B. Non-mammals

1. *Insects*

Although clearly recognized as less hazardous to nontarget organisms than chemical pesticides, some beneficial insects may be at risk from *Ma* exposure. Hajek and Goettel (2000) and Hajek and Butler (2000) provided guidelines and case studies on estimating damage to nontarget organisms. The basic approaches are labeled ''ecological host range'' as determined under field conditions and ''physiological host range'' ascertained under laboratory conditions. The ''physiological'' tests are deemed necessary, but experience has shown that ''ecological'' studies frequently indicate that field exposure is less hazardous than implied by the laboratory experiments. Integration of *Metarhizium* into diverse biological-control systems requires that the fungus be safe to parasites, predators, and pollinators (particularly in crops dependent on insect pollination).

Although stressed honeybees (outside their hives, at room temperature [$\sim$24°] and queen-less) are somewhat susceptible to *Ma-ac*, LC_{50} doses were 10 times that recommended for field use against locusts (Goettel and Jaronski, 1997). Honeybees held in cages at 28 °C and approx 80% RH were fed honey water with and without *Ma-an* conidia. Mortality was high due to high stress, but the rate of mortality was not enhanced by the presence of conidia (Allee and Roberts, unpublished). High doses (10^{10} conidia ml^{-1}) of *Ma-an* caused high mortality when sprayed on caged newly emerged adults (probably the most susceptible stage), but mortality was less with lower doses (Butt *et al.*, 1994). Nevertheless, fitting hives with *Ma-an* dispensers to cause foraging bees to spread the fungus to pest pollen beetles had no apparent adverse effects on honeybee colonies (Butt *et al.*, 1998). The finding of safety to hives is in agreement with that of Alves *et al.* (1996) who concluded *Ma-an* caused low mortality in treated hives, and had no adverse effects on behavior, larval development or colony characteristics—and that *Ma-an* probably will not cause epizootics in field hives following field applications of conidia.

Of crucial importance in integrated pest control is compatibility of the pest-control agent (chemicals or fungi) with the hymenopterous and hemipterous natural enemies (insect parasitoids and predators). The 500,000-ha *Ma-an* project to control spittlebugs in Brazil offers an excellent example of such compatibility. In sugarcane, the major pests are spittlebugs (*Mahanarva* spp.) (Homoptera) and stem borers (*Diatraea* spp.) (Lepidoptera). Many sugarcane plantations and grower

cooperatives produce annually tons of *Ma-an* for spittlebug control and millions of the hymenopterous parasite *Cotesia flavipes* for stem borer control, since *Ma-an* has very low virulence for *Cotesia* under field conditions. A similar situation, i.e. no infection of natural enemies, was noted in field trials with *Mf-pe* against lettuce root aphids (Milner, 1997). Also, two hymenopterous (Encyrtidae and Brachonidae) parasitoids of mealybug and Lepidoptera were exposed to *Ma-ac* in the laboratory, with resulting longevity reductions of up to 24% for the encyrtid, whereas parasitoids developed on infested plants held outdoors were not infected. The braconid was not susceptible to *Ma-ac* (Stolz *et al.*, 2002). Three *Ma-an* isolates with high virulence for coffee berry borer adults (Coleoptera: Scolytidae) also were virulent for a parasitoid (*Prorops nasuta*) (Hymenoptera: Bethylidae) in the laboratory (de la Rosa *et al.*, 1997), but one was less virulent to *P. nasuta* than the others (de la Rosa *et al.*, 2000). Introduction of the parasitoid 3–10 days after treating the coffee berries with this *Ma* isolate obviated parasitoid infection.

Grasshoppers in Australia parasitized in the laboratory by a nemestrinid fly had reduced susceptibility to *Ma-ac*, and the fly larvae were not infected by the fungus. Therefore the parasitic insect was expected to be active in maintaining low grasshopper populations, and the fungus could be employed in times of outbreak cycles without the severe parasitoid reductions noted with chemical insecticides (Milner *et al.*, 2002).

Beetles (Coleoptera), which are numerous and diverse in almost all field sites, were used as surrogates for nontarget insects in outdoor trials with *Mf* (*Ma-ac*?) isolates in Madagascar (Ivie *et al.*, 2002). One *Metarhizium* isolate had effects similar to those of a chemical treatment (fenitrothion + esfenvalerate) on nontarget beetle communities, but the other ''*Mf*'' (and a *B. bassiana*) had no detectable effects compared to untreated controls. This approach to safety studies allows observation of population responses in natural field (in contrast to laboratory) settings. As mentioned by Goettel and Hajek (2001), risk to nontargets may be overestimated by laboratory bioassays, and the findings in the field are important for estimating degree of risk or safety. The method of application can significantly affect laboratory studies. For example, conidia of two Finnish *Ma-an* isolates were highly pathogenic to an ichneumonid (Hymenoptera) parasotoid of rape blossom beetle (Coleoptera: Nitidulidae) when applied directly to prepupae in the laboratory, but in soil bioassays, the parasitoid was less affected than the pest species—leading to the conclusion that *Ma* is a useful candidate as a bio-insecticide for pollen beetle (Husberg and Hokkanen,

2001). Naturally occurring fungal infections in two families (Carabidae and Staphylinidae) of overwintering Coleoptera in several Danish fields revealed *Metarhizium* infected 0–2% of adults and approximately 12% of a small sample of larvae (Steenberg, 1995). The most common pathogen was *B. bassiana*, with adult infections of <10%, and higher levels in larvae—over 60% in one instance. Infection of these nontarget insects is speculated to assist in maintaining the fungi through winter. A coleopterous (Histeridae) predator of the larger grain borer (Coleoptera: Bostrichidae) proved less susceptible than its prey to *Ma-an* conidia in the laboratory, suggesting that the fungus may not be overly deleterious to the predator when used as a microbial control agent for the borer (Bourassa *et al.*, 2001). In a study that did not compare relative susceptibility to *Ma-an* of a coccinellid (convergent lady beetle) with that of its prey, first-instar larvae proved quite susceptible in laboratory bioassays (James and Lighthart, 1994). The LUBILOSA *Ma-ac* strain was not infective to two species of Tenebrionidae (Coleoptera), but two hymenopterous parasitoids were quite susceptible to 11 *Metarhizium* isolates in the laboratory (Danfa and Van Der Valk, 1999).

Laboratory screening of susceptibility to a Madagascar *Ma-ac* isolate indicated that none of four nontarget species in Coleoptera (Coccinellidae and Tenebrionidae), Neuroptera, and Aranae were significantly infected at doses which killed 100% of test locusts (Peveling and Demba, 1997). Outdoor nontarget tests with the LUBILOSA *Ma-ac* strain included Coleoptera (Carabidae and Tenebrionidae), Hymenoptera (Formicidae) and Diptera (Ephydridae). Carabids and ephydrids can feed on grasshoppers. Almost 30,000 specimens were collected in pitfall traps in this study. There was 75% reduction in nontarget insects when fenitrithion was applied, and 25% reduction following *Ma-ac* application. Accordingly, the fungal product was classified as ''low risk'' (Peveling *et al.*, 1999).

2. *Others*

A series of tests were conducted between 1994 and 1998 to determine possible adverse effects of *Ma-an* on fish, frogs, clams, and grass shrimp. Most of this work involved studies on embryogenesis of organisms exposed to conidia. There were no significant effects on larvae of the coot clam (*Mulinia lateralis*) (Gormly *et al.*, 1996), nor were there adverse effects on *Gambusia* fish fed freeze-dried *Ma-an* mycelium (Genther *et al.*, 1998). Methylene-chloride extracts of fungal cultures, which were assumed to contain *Metarhizium* toxins (destruxins), had LC_{50} values in grass shrimp (*Palaemonetes pugio*) and frog

(*Xenopus*) embryos of 50 and 30 parts per million, respectively. The fungal extract did not show mutagenicity in the Ames test. Significant mortalities were obtained with exposure of embryos of grass shrimp and silverside fish (*Menidia*) exposed to conidiospores, but there was no effect on frog embryos (Genther *et al.*, 1998). Doses administered in these studies were high compared to levels in water expected from field applications for pest-insect control, and dose-response reactions were absent in the fish-embryo experiments (Genther and Middaugh, 1995; Genther *et al.*, 1997). The results, therefore, constitute a ''worst-case'' scenario as indicators of safety risk.

VII. Strain Selection

In general, there is a paucity of potentially crucial information at the beginning of insect-control projects based on *Metarhizium*. Of key interest is which isolate(s) should be mass-produced for a given pest situation (Butt *et al.*, 2001b). This can be a daunting task, if for no other reason than that the number of isolates to select from is very large, and each step of the selection process can be time consuming (Bateman *et al.*, 1996). Ease of mass production is a consideration, but usually not a limiting problem with *Metarhizium*. Nevertheless, selection may hinge on strain differences when special needs, such as blastospore production, are involved.

A. Virulence

Laboratory virulence tests are not necessarily well correlated with field effectiveness. For example, a large number of fungal isolates were bioassayed in the laboratory in an effort to develop a fungus-based control system for the brown planthopper, *Nilaparvata lugens*, in Southeast Asia (Rombach, Aguda, Roberts; unpublished). Despite many variations in laboratory bioassay conditions, none of these isolates killed more than 70% of the brown planthoppers. Nevertheless, epizootics of *Mf-minus* and *Hirsutella citriformis* routinely occurred in the field. Fungal isolates with very low infectivity to the target host in the laboratory, of course, should not be developed further. On the other hand, there is always the haunting possibility that mediocre performance in the laboratory may mask a truly potent pathogen – one which might warrant further development. Mistakes also can be made in the other direction, namely a fungus (and this is more often the case) may be extremely virulent to the target pest under laboratory conditions and

yet fail in the real world of field use. An antibody raised to conidial surface antigens of a virulent *Mf-fl* isolate was used to index other isolates (Rath *et al.*, 1996). Virulence of the isolates correlated with their antibody indices, suggesting that an *in vitro* test for virulence is possible.

B. Environment/Habitat

In addition to virulence, the isolate also must be ''in tune'' with the habitat of the target insect(s); and, in fact, selection in nature of *Metarhizium* may be as much as on environmental factors as on specific hosts. This includes temperature and tolerance to solar (UV) irradiation (Bidochka *et al.*, 2001b; Braga *et al.*, 2001c; De Croos and Bidochka, 1999; Fargues *et al.*, 1992, 1996; Rath *et al.*, 1995; Thomas and Jenkins, 1997; Yip *et al.*, 1992), as well as physiology, e.g. high cuticle-degrading-protease production (Pinto *et al.*, 2002). The effects of solar UV on fungi, as well as methods for study of this topic, are discussed by Braga *et al.* (2002) and Roberts and Flint (2002). Sunlight contains UV-A (315–400 nm) and UV-B (290–315 nm), but atmospheric ozone excludes UV-C (100–280 nm). UV-B, with its direct action on DNA, is considered more biologically active than UV-A, which is oxidative in nature. *Ma-an* isolates were susceptible to both solar wavelengths, but considerably more so to UV-B than UV-A (Braga *et al.*, 2001c). The half-life of conidia in full-spectrum sunlight was less than three hours. for two *Ma-an* isolates (#23 and #2575) (Braga *et al.*, 2001c). There was a relationship between equatorial location (latitude) of isolate origin and degree of UV-B tolerance, with *Ma-ac* (especially ARSEF #324, and to a lesser extent the LUBILOSA isolate #IMI330189) being much more tolerant than the 20 *Ma-an* isolates tested (Braga *et al.*, 2001d; Rangel, Braga, Roberts, unpublished). Exposure to UV-B induced delayed germination of conidia, which may reduce pest-contcol efficacy (Braga *et al.*, 2001c). Both conidia and germ tubes were susceptible to UV-B (Braga *et al.*, 2001a,b). *Ma-ac* (#324 and #3609) exhibited considerably greater tolerance to heat (45 °C) than 15 *Ma-an* isolates tested (Rangel, Braga, Anderson, Roberts, unpublished). Although the occurrence of extreme temperatures in insect habitats is common knowledge (e.g. less than 10 °C in Tasmanian soil and more than 40 °C in semitropical solar-exposed soil surfaces), screening of natural isolates usually is incorrectly focused almost exclusively on virulence.

C. Soil Adaptation (Rhizosphere Competence?)

1. *The ''True Nature'' of* Metarhizium

The preferred natural habitats of *Metarhizium* spp. remain unclear (Prior, 1992). Although *Metarhizium* spp. can be recovered from soil worldwide by direct plating on selective media (Goettel and Inglis, 1997), it is not clear whether what is being recovered are conidia, mycelia surviving on insect remains, or mycelia living on non-insect substrates. Likewise, it is not known if these fungi genuinely flourish in soil or whether they survive there in a dormant state awaiting a susceptible host (Inglis *et al.*, 2001; Prior, 1992). Conflicting results may derive from genetic diversity among *M. anisopliae* strains in conidial survival and from the levels of fungistasis in different soils. Perhaps only some strains can grow saprophytically in soils (Mikuni *et al.*, 1982); and, exceptionally, these may form extensive rhizomorphs (Prior, 1992). Such capacity could be linked to the ability to secrete antimicrobials against many common saprophytes (Chul *et al.*, 1996; Walstad *et al.*, 1970). The great majority of *Metarhizium* isolates available are from insects, but this mainly reflects the interests of collectors. Aside from a report that many soil isolates are non-pathogenic to scarab beetles (Milner, 1992), there is little information available on the relative virulence of isolates of fungi from soil and from insects. It may be that there are two diverse sets of selection pressures on entomopathogenic fungi frequently isolated from soil (e.g., *Metarhizium* spp. and *Beauveria* spp.): one for optimum characteristics for soil survival and another for virulence to insects (Prior, 1992). If so, it is unlikely that the same characteristics will be optimum for both insects and soil. Thus Bidochka *et al.* (2001b) showed that genetic groups of *M. anisopliae* were linked to habitat type rather than to insect host—for example, isolates from open-field habitats belonged to a different group than forested-soil samples. They concluded that selection in nature for the saprophytic soil-dwelling phase is more important in shaping the population genetics of *M. anisopliae* than is selection for the pathogenic phase.

Rhizosphere competence is particularly important when considering the potential commercial use of biocontrol agents toward soilborne plant pathogens (Baker, 1991), and presumably the same could apply to pathogens of root insects. A recent comprehensive review article on biocontrol in the rhizosphere, however, made no mention of fungal-insect interactions while describing interactions between fungi and protozoa, bacteria or other fungi (Whipps, 2001). Sylvia (1998) defines ''rhizosphere'' as the zone of soil immediately adjacent to plant roots in

which the kinds, numbers, or activities of microorganisms differ from that of the bulk soil, and ''rhizosphere competence'' as the ability of an organism to colonize the rhizosphere. General surveys employing selective media have shown that *M. anisopliae* is ubiquitous and that most cultivated and non-cultivated soils contain between 2×10^3 and 10^6 propagules/g (e.g., Milner, 1992). The fungus is most abundant in undisturbed pastures in soils 2–6 cm deep, where it commonly exceeds 10^6 propagules/g (Milner, 1992). In our studies at Upper Marlboro, Maryland, results were similar (Hu and St. Leger, 2002). Indigenous *Ma-an* was found at its lowest levels in cultivated sites ($<10^3$ fungal propagules/g) and at highest levels (10^6 fungal propagules/g) in soils 1–5 cm deep from fallow (pasture) land. Such large microbial soil populations are normally the result of organic substrates in rhizospheres of the upper layers of the soil (Dix and Webster, 1995). Given that rooting density is particularly high in grasses and cereal crops, i.e., <3 mm spaces between roots (Barley, 1970), the flourishing *Ma-an* community must be living in overlapping rhizospheres. Presumably a large population of insect hosts could also contribute to these *Metarhizium* populations, assuming that the soil strains are pathogenic.

The origins of insect pathogenic fungi also could provide clues to their abilities. They may have evolved from endophytes of plants or from plant pathogenic fungi (themselves descendents of endophytes?) (St. Leger and Bidochka, 1996). *M. anisopliae* clusters with clavicitaceous grass endophytes (*Epichloe*) in phylogenetic studies (Sung *et al.*, 2001). *Metarhizium* and *Epichloe* produce very similar proteases at very high levels during interactions with their hosts (Reddy *et al.*, 1996), and *M. anisopliae* can penetrate some leaf surfaces after germinating and forming appressoria against them (Inyang *et al.*, 1999). The equally well-known insect pathogen *B. bassiana* is a vascular endophyte of corn, functioning as a protective mutualist (Wagner and Lewis, 2000). Although clavicipiteous endophytes are said not to extend into roots (Carroll, 1992), the possibility that *B. bassiana* may do so has not been investigated. A strain of *M. anisopliae* parasitizes plant cyst-nematodes, as well as beetle larvae (Carris and Glawe, 1989), but it is not clear whether the fungus penetrated the cyst from outside or inside the root (Stefan Jaronski, personal commication). Surveys of endophytes in Brazil have failed to detect *Metarhizium* (Azevedo *et al.*, 2000; Azevedo, personal communication). Keller and Zimmerman (1989) concluded a review on the mycopathogens of soil insects with some pertinent questions: How far is fungal saprophytic life possible? Does saprophytic ability vary only between species, or also between

strains? How does the fungus spread? Are there possibilities for improving the rate of multiplication and survival? Answers to these and similar questions are of great fundamental and practical interest with respect to soil-insect-mycopathogen relationships. Clearly there is much to learn about the biology of *Metarhizium;* fortunately, the ability to incorporate molecular tags such as the *gfp* gene into pathogen strains will make analyzing population dynamics in soil feasible (Hu and St. Leger, 2002). (See VIII. C)

2. *Relevance of Rhizosphere Competence to Biocontrol*

As mentioned previously, most biocontrol studies employing *Metarhizium* spp. have selected strains for optimum virulence against pest insects and have ignored habitat preferences and survival outside the host. With soil insects, searches for highly virulent isolates of this fungus may be inherently flawed, given that factors associated with soil-dwelling may be even more critical in the selection of an isolate than virulence *per se* (Bidochka *et al.*, 2001b). In the case of strains engineered for improved virulence, such strategies may fail if genes are engineered into a strain that survives poorly in a certain habitat. On the other hand, there are other environmental and economic reasons why researchers and industry would not seek to permanently establish an engineered microbial agent in the environment (St. Leger and Screen, 2001), and rhizosphere competence might increase the difficulty of eliminating the pathogen following unanticipated and deleterious environmental effects. Evidently, a key to achieving successful, reproducible, and safe (from the risk management point of view) biological control lies in detailed knowledge of the properties of pathogens in their environments. If rhizosphere competence is a general phenomenon among *Metarhizium* isolates, its impact on plant ecology could be considerable—with implicit co-evolutionary implications—and then should be considered in selecting fungal strains for biocontrol. This also raises the possibility of managing the rhizosphere microflora to achieve insect control, which dovetails with attempts in IPM to manipulate the environment of the plant and insect to enhance insect biocontrol (Roberts and Hajek, 1992). If a good root colonizer *Metarhizium* isolate is available—viz. one that is capable of being transported by the root through the soil profile—then seed treatment would be an attractive method for introducing the fungus into the soil-plant environment where it may have the opportunity to be the first colonizer of roots. However, such attempts are more likely to fail if the process of root colonization is poorly understood. An understanding of the nature

and function of microbial gene products expressed during growth in soil and on root exudates is an essential step in attaining a molecular explanation for the ''fit'' between the fungus and its environment.

D. Stability of Strains

Fungi can be unstable genetically, and vigilance must be exercised to guarantee that virulence and the ability to sporulate remain high (Kamp and Bidochka, 2002; Ryan *et al.*, 2002). The selected isolate should be passed through the target host, multiplied, and stored in many small aliquots. Each, or almost each, production run should start from this stock. Some sugarcane plantation cooperatives in Brazil commence each production run with a fresh isolate from a field-collected sick insect. This probably protects virulence, but obviates having a ''standard strain'' with known safety and production characteristics. Preservation of isolates in a stable form is very important (Humber, 1997). Cryopreservation and lyophilization disrupted secondary-metabolite production in one of three *Metarhizium* isolates tested, and changed polymorphisms in two out of three isolates examined by PCR fingerprinting (Ryan *et al.*, 2001). Accordingly, survival does not guarantee genetic stability. Mineral oil over agar cultures in small plastic test tubes held at 4 °C is an effective approach to genetically stable storage since cultures live for decades without transfer, can be sampled repeatedly, and no special equipment is needed. The tubes must be maintained upright, however to prevent spilling and contamination. Backup cultures should be maintained in a liquid nitrogen-based facility, if practicable.

VIII. Strain Improvement

A. Virulence Traits

In addition to inconsistent results, the slow speed of kill of biologicals compared with chemicals has deterred commercial development. For example, it usually takes 10–20 days for *Ma-ac* (''Green Muscle'') to significantly reduce locust populations, and this is constraining successful commercialization (Hajek *et al.*, 2001). Consequently, consideration of a pathogen for commercial development inevitably leads to discussion of improving its performance (Harrison and Bonning, 1998); and this necessitates detailed knowledge on which traits are important to virulence.

1. *Specific-gene Analysis*

Current knowledge on virulence traits comes primarily from studies on the function of specific genes and gene families in the pathogenicity of *Ma-an* strain ARSEF #2575 (=Me1) (Joshi and St. Leger, 1999; Screen and St. Leger, 2000; St. Leger and Joshi, 1997; St. Leger *et al.*, 1992a,b). This research demonstrated that host-pathogen interactions, including formation of infection structures and secretion of cuticle-degrading enzymes, are inducible by physical and chemical signals (St. Leger, 1993). Induction involves a complex interaction of many factors, including protein phosphorylation events, transcriptional regulatory factors and physiological cues within the fungus (see reviews by St. Leger and Bidochka, 1996; St. Leger and Screen, 2001). Addition and expression of pesticidal genes in *Ma-an* isolates are straightforward and can be used to improve pathogen performance (St. Leger, 2001a). In fact, a strain has been genetically engineered to over-express toxic proteases and thereby kill insects 25% faster (St. Leger *et al.*, 1996).

2. *Expressed Sequence Tag (EST) Analysis*

Attempts to discern key physiological determinants of host/pathogen interactions by focusing on the structure and function of specific *Metarhizum* genes and gene families can be thwarted by the complexity of the microbial responses to host-related signals. Thus side effects occurring in selected or constructed strains are difficult to predict and assess. Lack of knowledge of inter-related regulatory and metabolic processes in cells inhibits exploiting the full range of engineering possibilities. Accordingly, an expressed sequence tag (EST) strategy (i.e., partial sequencing of randomly selected cDNA clones) was adopted as a cost-effective way to assess *Ma-ac* (ARSEF #324) and *Ma-an* (ARSEF #2575) strains as ''whole systems'' rather than as isolated parts. Because the libraries were made from fungi growing on insect tissues, the EST database (http://tegr.umd.edu) represents the subset of #324 and #2575 genes active during infection processes (''insect-response genes''). Among them are genes coding for metabolic and biosynthetic pathways and regulatory functions, as well as other genes that could conceivably affect virulence (Freimoser *et al.*, 2002). Of particular note are many homologs with *Streptomyces* (bacterial) proteins, including several classes of protease, catalase, esterase, and toxins. Arguments for trans-kingdom horizontal gene transfer of one such protein were recently detailed (Screen and St. Leger, 2000). ESTs have allowed cloning of many activities previously only identified

by biochemical studies and have extended the families of target proteins—for example, several new genes encoding peptide synthetases that produce destruxins (insect-specific toxins). These studies are rapidly increasing the range of tools available for biotechnology. ESTs also are revealing new, unsuspected stratagems of entomopathogenicity and biologically active molecules. For example, *Ma-an* #2575 secretes previously undescribed toxins and a cocktail of enzymes into its host that resemble the constituents of some snake venoms (Freimoser *et al.*, 2002; St. Leger and Screen, 2000).

Of particular interest is an EST-based comparison of a generalized (wide-host range) strain with a specialized (narrow host range) strain, viz. *Ma-an* #2575 vs *Ma-ac* #324, respectively (Freimoser *et al.*, 2002). Strain #2575 has genes for multiple classes of enzymes/toxins; and, in fact, *ca.* 60% of its ESTs encode secreted enzymes and toxins (e.g., enniatin, versicolorin B, phenazine, destruxins, etc.). Acting collectively, the number and diversity of these effectors may be the key to this pathogen's ability to infect a wide variety of insects. In addition, insertion elements found among the ESTs could promote genetic instability by increasing the frequency with which a DNA sequence is gained or lost. This suggests that evolution may occur in leaps, which may have consequences for product stability. In contrast, ESTs from #324, the specific *Ma-ac* acridid pathogen, revealed very few toxins (Freimoser *et al.*, 2002). This relates to life-styles. *Ma-an* strain #2575 kills hosts quickly via toxins and grows saprophytically in the cadaver. Strain #324, however, causes a systemic infection of host tissues before the host dies. Utilizing ESTs allowed simultaneous viewing of multiple virulence factors and pathways, thereby permitting a broader understanding of the different lifestyles existing in insect-fungus interactions.

The variability and redundancy found in *Metarhizium* genomes present major challenges to understanding the fungus ecology strictly by considerations of homology and function. EST analysis showed that *Ma-an* #2575 produces 11 subtilisins, the largest number from any fungus. PCR was used to obtain their orthologs (counterparts) from *Ma-an* ARSEF strain #820 and *Ma-ac* #324 (Bagga, Screen, and St. Leger, unpublished). Diversification is an ongoing process. Thus, Pr1E and Pr1F subtilism genes were produced by tandem gene duplication from a Pr1F-like ancestral gene after divergence of #2575 and #324 (Pr1E is missing in #324 and in tandem in #2575 and #820). Comparing multiple paralogs (isoenzymes) from the three strains showed that most amino acids were under selective constraint for all 11 subtilisin proteases—that is, each protein makes a significant selectable contribution

to pathogen fitness. Sequence comparisons and homologous modeling using the known 3-D structure of proteinase K from *Tritirachum album* were used to predict that the subtilisins are all rather unspecific enzymes, but that they will differ in their interactions with protease inhibitors, secondary substrate specificities, absorption properties and alkaline stability, consistent with functional differences that allow the proteases to act synergistically for more efficient hydrolysis of cuticle. Multiple proteases may also provide backup systems in the presence of the numerous proteolytic inhibitors described in insect hosts (Boucias and Pendland, 1997). The evolution of host specificity includes a race between pathogen virulence traits and host defenses.

3. *Microarray Analysis*

Changes in mRNA expression (''up-regulation'' or ''down-regulation'') can be monitored by hybridizing mRNA to DNA; and this is most effectively accomplished by using microarray. Although there is not always a direct correlation between the expression level of an mRNA and expression of the protein for which it codes, the likelihood of a link provides valuable indications of the multiple interactions of genes during fungal responses to their hosts and/or to stresses. Microarray analysis is fast, user friendly and convenient. The non-radioactive, non-toxic hybridization solution and the comforting knowledge that the arrays are inexpensive and easily replaced has facilitated exploratory experiments to achieve the long-term goal of determining the role of the key genes involved in an insect pathogen's response to an insect host. This is feasible only if the experiments are organized into a hierarchical approach based on grouping genes of related function. To achieve this, about 3500 EST unigenes of strain ARSEF #2575 were microarrayed and then categorized using software packages for cluster analysis according to time of expression during the pathogenic phase and/or to induction by external physical or chemical host-related stimuli. These data were displayed graphically to discern patterns of gene expression on a broad scale (overview of the choreography) and fine scale (gene by gene). This proved very informative as to gene function, since numerous clusters of co-expressed genes which represent diverse expression patterns were strikingly coherent in their cellular functions. Thus, a cluster of ribosomal proteins and other genes (such as elongation factors) involved in translation were down regulated during early growth on cuticle. At the same time, more than 300 genes were up-regulated, indicating that the fungus relies on preformed machinery for their translation. Many of these are as yet uncharacterized genes, and most have never been recognized to have a

role in pathogenicity. These novel genes, however, are already implicated by co-regulation with known virulence factors, and invite further characterization to establish their functional role(s). Other genes have homologs across a range of genera, whereas some have homologs only in plants and bacteria. The latter include many antimicrobial molecules, e.g., anti-fungal thaumatins, that may be up-regulated as an adaptation to allow *Ma-an* to defend limited resources within the insect cadaver.

The huge amounts of data accruing from the microarray analyses have shown that various aspects of adaptation to growth on insect cuticle, e.g., enzyme production, are associated with up regulation of a large number of genes encoding components of signal transduction. Through these, the fungus is in continual communication with its surroundings to regulate morphological infection processes. Genes encoding components of membrane biogenesis, synthesis of cell wall components, storage or mobilization of nutrient reserves and protein folding also are highly expressed, indicating manufacture and ''remodeling'' of cell structures. Another feature of *Ma-an* physiology highlighted by this work is the need to take up and absorb peptides and amino acids from the cuticle. This is illustrated by the very early induction of a variety of transporters and permeases following contact with the cuticle. Early uptake of peptides allows the fungus to ''sample'' the cuticle and results in induction of proteases. The 11 known *Ma-an* subtilisins provide one of many examples where different members of a family are differentially regulated. Likewise some hydrophobins (cell-wall proteins) are regulated during early appressorial development, whereas others appear later—which is consistent with different functions. Except for the trypsins, protease production is sharply down-regulated in culture media containing hemolymph. It may be relevant to this that the trypsins have a secondary specificity (St. Leger *et al.*, 1987a,b,c) very similar to that of bacterial immunosuppressive proteases (i.e. they degrade antimicrobial peptides) (Caldas *et al.*, 2002) and unlike the subtilisins, do not activate *M. sexta*'s prophenoloxidase system (St. Leger *et al.*, 1996).

B. Improving Virulence (Speed of Kill)

Transformation systems for *Ma-an* are well established (Bernier *et al.*, 1989; Goettel *et al.*, 1990b; Smithson *et al.*, 1995; St. Leger *et al.*, 1995). A strategy was devised to introduce depolymerases and toxins in such a way that would increase speed of kill. Since activation of fungal infection processes involves the expression of many

inducible proteins, constitutive expression provides a direct strategy for engineering enhanced virulence. This may over-ride effects produced by physical and chemical signals that induce a transient expression of actions of the gene. The most attractive initial candidates for this approach include genes encoding cuticle-degrading enzymes and toxins, as these have often been found to be active synergistically *in vitro* against insects (St. Leger, 1993); and, since the active agents are encoded by single genes, they should be highly amenable to manipulation by gene transfer.

We used this strategy to develop the first genetically improved entomopathogenic fungus (St. Leger *et al.*, 1996). Additional copies of the gene encoding the cuticle-regulated Pr1a protease were inserted into the genome of *Ma-an* ARSEF #1080 under the control of a constitutive promoter such that the gene was constitutively expressed. In contrast to the wild type, the transgenic strains continued to produce Pr1 in the hemocoel of *Manduca sexta* caterpillars following penetration of the cuticle. This caused extensive melanization in the body cavity and reduced time to death by 25%. Extensively melanized cadavers were very poor substrates for growth and conidiogenesis of the fungus, and thereby provided a degree of biological containment (St. Leger *et al.*, 1996).

C. Field Testing Transgenic Strains of *Metarhizium*

Although very significant amounts (up to 60%) of certain field crops used for animal feed in the U.S.A. are genetically engineered, uncertainty remains in some circles as to the safety to the environment of transformed plants and microorganisms (Caprio *et al.*, 2000). In the summer of 2000, to examine the risk of long-term establishment of genetically altered insect pathogenic fungi, we (RSL) released onto a plot of cabbages at a field site on the Upper Marlboro Research Station, Maryland, two transformed derivatives of *Ma-an* #2575 (Hu and St. Leger, 2002). The transformants carried the *Aequorea victoria* green fluorescent protein (*gfp*) gene alone (GMa) or with additional protease genes (*Pr1*) (GPMa). The study: (a) confirmed the utility of *gfp* for monitoring pathogen strains in field populations over time; (b) demonstrated little dissemination of transgenic strains and produced no evidence of transmission by non-target insects; (c) found that recombinant fungi were genetically stable over one year under field conditions; and (d) determined that deployment of the transgenic strains did not depress the culturable indigenous fungal microflora. The major point of the study was to monitor fate (survivorship) of transformants under

field conditions. In non-rhizosphere soil, GMa decreased from an initial 10^5 propagules/g at depths of 0–2 cm to 10^3 propagules/g after several months. However, densities of GMa remained at 10^5 propagules/g in the inner rhizosphere, demonstrating that rhizospheric soils are a potential reservoir for *M. anisopliae*. This contrasts with most studies since even good root colonizers usually show a decline in numbers at the rhizosphere, perhaps because the initial population added is too large for the carrying capacity of the root (Parke, 1991). These results place a sharp focus on the biology of the soil/root interphase as a site where plants, insects and pathogens will interact to determine fungal biocontrol efficacy, cycling and survival. The rhizospheric effect was less marked for GPMa; and, overall, it showed reduced persistence in soils compared to GMa.

The results of the outdoor trial did not suggest any safety concerns to using GMa or GPMa that would detract from their being environmentally preferred alternatives to chemical pesticides. Their survival into the second year is significant, since time may increase the possibility of adaptation for increased fitness. It cannot be assumed, therefore, that either strain will die out because of current reduced fitness. There was no evidence for phenotypic instability of the introduced fungi; but this might not be expected if genetic changes with clear phenotypes follow the punctuated equilibrium model of evolution; i.e., long periods of apparent stability, punctuated by large, infrequent changes. Given that the long-term fitness of a genetically engineered pathogen that persists in nature is difficult to predict, it is all the more essential to establish technologies, such as *gfp*, that permit informed risk assessment through following the fate of marked strains.

IX. Mass Production

The discoverer of *M. anisopliae*, Elias Metschnikoff, suggested the fungus could be used to control a serious pest, the sugar-beet curculio, *Bothynoderes* (= *Cleonus*) *punctiventris*. Realizing that large numbers of spores were required, he demonstrated that *M. anisopliae* conidia could be produced on sterilized beer mash (Metschnikoff, 1880). Although he obtained encouraging preliminary field results (Steinhaus, 1975), his interests were diverted to other problems in Italy where he discovered the phenomenon of phagocytosis. Nevertheless, he apparently inspired a Ukranian, Isaak Krassilstschik (1888), who in 1884 produced 55 kilograms of ''absolutely pure'' spores in four months. These were mixed with sand and applied to several fields near Kiev at the rate of 8 kg/ha. Two weeks later larval mortality ranged

from 55 to 80%, with green spores on the cadavers. He claimed the factory, labor, and substrate were very inexpensive; and time was the principal factor in spore production. The project was not pursued further, however. Difficulties in producing *M. anisopliae* described in detail by Krassilstschik are woefully similar to those expounded to DWR by some new *Metarhizium* factories in Brazil in 2002. In particular, sterilizing large amounts of substrate is not a trivial activity; and shortcuts taken to save time or energy result in unacceptable levels of contamination from other fungi and spore-forming bacteria.

Since mass production and formulation are key to successful inundative use of entomopathogenic fungi, both as to effectiveness and cost, interest in these subjects has persisted the past 120 years (Bateman and Chapple, 2001; Feng *et al.*, 1994; Jenkins and Goettel, 1997; Wraight and Carruthers, 1999; Wraight *et al.*, 2001). It should be noted that the details of commercial production systems are seldom published. Non-commercial organizations sometimes afford refreshing exceptions: e.g. the detailed instructions of LUBILOSA, including quality assurance procedures (Jenkins *et al.*, 1998); the floor plans and equipment lists published in Brazil to encourage individual sugarcane plantations to produce *Ma-an* conidia (Alves and Pereira, 1998); and although never built, a system was devised from combining parts of several production schemes (Burges, 1998b).

Because almost all isolates of *Metarhizium* will grow and sporulate on very simple media such as rice, rice bran, and virtually all microbiological media, it is easy to think that spore production is a readily solved problem when developing insect control programs based on conidia as the delivery inoculum. There are, however, a number of pitfalls in producing large amounts of the fungi. As already mentioned, sterile methods must be adhered to very strictly, since many organisms can outgrow, or kill, *Metarhizium*. For example, one production facility in Brazil became contaminated with a *Penicillium* which rendered their *Metarhizium* conidia non-viable, perhaps due to patulin produced by the *Penicillium* (Lingg and Donaldson, 1981; Shields *et al.*, 1981). Simple laboratory procedures which afford sufficient numbers of conidia for small-scale field trials can be very expensive if simply expanded without serious modification. Space can become a limiting factor when, for example, many small bags of inoculated rice must be incubated for two or three weeks. When the substrate containers are too crowded, or the layer of substrate in which the fungus is growing is too deep, the heat of metabolism from the growing fungus also can be an important limiting factor (Dorta and Arcas, 1998). Moderate-scale production facilities for *Beauveria bassiana* in China (PRC) have used

trays (wooden baskets) with a layer of rice bran no thicker than approximately 40 to 50 mm and with a moist-cloth covering. The trays are mounted on racks where there is a great deal of air circulation. Another PRC approach to controlling heat is to grow the fungus in shallow depressions in the soil, thus using the soil as a heat sink. *Beauveria* is very competitive, and if given a head start it can often stop the development of contaminating microorganisms. *Metarhizium*, unfortunately, is not as competitive and does not produce the same antibiotics—primarily, oosporein (Roberts, 1981)—of *Beauveria*. Labor can be a limiting factor if the production scheme requires a great deal of handling of small units. Because the entire operation seems so simple, people with little or no microbiology training establish production facilities, or factory owners employ quite inexperienced personnel. Large-scale (commercial-scale) production will require at least some sophisticated equipment.

Virtually all large-scale production of *Metarhizium* conidia is done on vegetable products such as rice or rice bran. The ''starch-based'' solid-substrate production facility built by MycoTech (now Emerald BioAgriculture) uses a large computerized fungus-production chamber in Montana which cost several millions of dollars. Although they currently have only *B. bassiana* products, their facility presumably could be adapted to *Ma* production. Conidia can be used as inoculum, but the time from inoculation of the grain to harvest of spores will be shortened several days by commencing the process with fungal mycelium in the log phase of growth. Accordingly, small production facilities often use shaker cultures, and large ones purchase fermenters (up to 300 liters) to produce the inoculum. Large-scale harvesting of the product offers opportunities for excessive exposure of personnel to conidia. Production costs and quality control of infective units are limiting factors in the field-use of *Metarhizium* (Jenkins and Grzywacz, 2000).

A. Approaches to Mass Production

Great creativity has been applied to producing, harvesting, formulating, and introducing mass-produced *Metarhizium*. Of major importance is determining for the selected fungal strain the proper mass-production system—for example, optimum substrate, temperature for incubation, proper time to harvest, and effective conditions for storage—including moisture content, oxygen availability, temperature and type of packaging. The final products are mycelium, blastospores or conidia.

1. *Mycelium*

Submerged culture, such as shake flasks or fermenters, is the system recommended by the fermentation industry for efficient production of fungal biomass. In general, however, fungi do not conidiate in submerged culture—spores usually are the infective units for insect control. The fermentation industry normally grows fungi for the production of chemicals such as antibiotics, enzymes, acids, and other commercially valuable fungal metabolites; and, therefore, this industry prefers to discourage conidiation. Nevertheless, to take advantage of the large biomass available through fermentation, insect pathologists have attempted to use mycelium for insect control. The objective has been to place small aggregates of viable mycelium in the field with sufficiently high-humidity conditions for spores to be produced on the clumps. Mycelium in fermentors can become so viscous after vigorous growth that aeration is seriously impeded, and it then undergoes a very rapid and nearly complete autolysis. Accordingly, harvesting must be done before this concentration occurs. A system to store mycelium in a viable state was devised (McCabe and Soper, 1985), and the product was referred to as ''dry mycelium.'' Mycelium was produced in air-lift fermentors (liquid medium vigorously agitated with air) until peak growth, the medium removed with a cloth filter, washed with water, the layer of mycelium stripped from the cloth, placed on racks, sprayed with a maltose solution and held overnight at 4 °C—after which it was dried in a stream of air for several hours until ''crisp.'' The first experiments were with Entomophthorales fungi, but the system has been adapted to a number of imperfect fungi, including *Metarhizium* spp. (Magalhaes *et al.*, 1994; Pereira and Roberts, 1990; Rombach *et al.*, 1986d). Unlike Entomophthorales and *Culicinomyces*, *Ma-an* survived drying without a sugar spray (Pereira and Roberts *et al.*, 1987). The final product is milled to particles of approximately 0.5 mm, preferably with a milling system that does not generate heat. Mycelium also has been formulated with sodium alginate or pre-gelatinized starch prior to drying (Marques *et al.*, 1999; Pereira and Roberts, 1991). The *Metarhizium* product, before or after milling, is stored at −20 ° or 4 °C but without special packaging, it does not survive more than a few weeks or months. Mycelium of two entomopthorales fungi vacuum packed in plastic with silica gel and an oxygen scavenging material survived for at least three months at 23 °C (Leite, 2002). Rupturing of some cells during drying or rehydration was proposed as a source of nutrients for conidia production on dry mycelium particles (Goettel *et al.*, 1989).

In outdoor trials in Asia, reasonably high levels of control were obtained by spraying with large-orifice nozzles dry-mycelium particles of several species of Hyphomycetes to control the brown plant hopper (Aguda *et al.*, 1987; Rombach *et al.*, 1986a,b,c). High humidity (preferably rainfall) was necessary for sporulation after application, although periodic rain followed by desiccation still allowed sporulation. Soaking the mycelium prior to field application did not enhance the levels of brown planthopper control. In other experiments two problems were noted: dry mycelium in soil was found to be repellent to Japanese beetle larvae (Villani *et al.*, 1994), although conidia were not repellent to this insect species. Possibly the repellency of mycelium was specific to certain *Metarhizium* isolates. Secondly, because the mycelium had been sprayed with maltose, particles sometimes were scavenged by ants before they conidiated.

An isolate of *Ma-an* designated ''BIO 1020'' was produced in two-step liquid culture as small mycelial colonies (''pellets'') approximately 0.5–1.0 mm in size. These were dried, vacuum packed, and stored at 4–20 °C for up to several months (Andersch, 1992; Andersch *et al.*, 1990). The pellets were mixed with soil for production of conidia and control of black vine weevil larvae, *Otiorynchus sulcatus* (Hartwig and Oehmig, 1992; Stenzel, 1992; Storey *et al.*, 1990). The product was registered in Germany, by Bayer; but it was not aggressively merchandized. The data presented at several meetings in the early 1990s indicated that this was an effective product. It is currently being developed in the United States by a new company, Earth BioSciences, in New Haven, Connecticut. They will produce the fungal strain on a grain ''carrier'' (Wells, personal communication). The spore-covered grains will be applied against soil insects, ticks, and greenhouse-house pests (particularly mites). A single application is expected to be adequate for a full growing season due to continued spore production on the grain in the field.

2. *Blastospores and Submerged Conidia*

There have been a number of successes in producing spores in submerged (liquid) culture. Several media extant were designed to encourage Hyphomycetes, including *Metarhizium* spp., to produce blastospores (Fargues *et al.*, 2002; Kleespies and Zimmermann, 1998). Blastospores are pieces of mycelium infective to insects. There are no commercial products at present based on *Metarhizium* blastospores, but there has been some success with desiccation-tolerant blastospores of *Paecilomyces* (Jackson *et al.*, 1997). Improved storage (~3 mo.) was obtained with dry *Ma-ac* blastoconidia by adding sucrose to the medium

at 96 hrs and hydrated silica at harvest (Quimby Jr. *et al.*, 2001). Nevertheless, this type of spore is not considered ideal for field use since the thin cell wall is more susceptible than conidia to desiccation and solar irradiation on leaves. Rare species and/or strains of fungi produce conidia as well as blastospores in submerged culture, e.g., an isolate of *Ma-ac* (Jenkins and Prior, 1993). These conidia are less heavily walled than those produced on solid substrates, and their tolerance to harsh environmental conditions is less than that of aerially produced conidia. They apparently lack the hydrophobins found on the surface of aerial conidia. These compounds are extremely important in attachment of conidia to the hydrophobic surface of insects (Bidochka *et al.*, 2001a; Jeffs *et al.*, 1999; St. Leger *et al.*, 1992b, 1998). Suspension of *M. anisopliae* blastospores and submerged-culture conidia in 20% skimmed milk powder permitted these spores to survive spray-drying with little loss of viability (Stephan and Zimmermann, 1998).

3. *Aerial Conidia*

Aerial conidia, in general, are the preferred units for wide-scale application of *Metarhizium* for pest control (Wraight and Carruthers, 1999). Fungi normally produce mycelium on solid substrates until nutrients become limiting, as evidenced by high *Ma-an* conidial counts on very thin (2-mm) potato dextrose agar (Kamp and Bidochka, 2002). As mentioned earlier, mass production is usually based on some type of vegetable substrate. For example, almond mesocarp (a common waste product in Mediterranean countries) (Lopez-Llorca *et al.*, 1999) and various legume seeds (Vilas Boas *et al.*, 1996) have been proposed as solid substrates. Rice bran and wheat bran are sometimes used commercially; particularly when mixed with rice husk and with the moisture content carefully adjusted, they afford high spore yield (Dorta *et al.*, 1990, 1996; Magalhaes and Frazao, 1996). The most frequently employed substrate, however, is rice grain. The grains afford a large surface area and allow circulation of gases. A very popular type is ''broken rice,'' which is considered a waste product by rice mills, and therefore is inexpensive (Quintela, 1994). Also, the small-size grains afford large surface area per weight; although its smaller fragments can make harvesting conidia more difficult (Magalhaes and Frazao, 1996). A key step is the removal of glutens from the rice to prevent grains from adhering (''rice pudding'') and thereby preventing circulation of air between the grains. Gluten removal normally is done by washing the rice in hot water (parboiling) or by using non-glutenous rice (indicus), and the containers manipulated after autoclaving to be certain that the

grains are not adhering. The rice may be purchased already parboiled, or boiled and drained and then placed into bags or trays. The Bio-Care (Australia) production scheme utilizes parboiled broken rice, while LUBILOSA in Benin uses unbroken rice supplemented with a small amount of peanut oil (Jenkins *et al.*, 1998). Burges (1998b) suggests use of buckwheat to obviate aggregation of grains. Culture substrate also affects storage capability and virulence of conidia (Daoust and Roberts, 1983b), and media influenced the germination of conidia produced on mycelium of three strains of *Metarhizium* (Ibrahim *et al.*, 2002). Three media were tested, one of which was supplemented with KCl to increase osmolarity. In addition, a second set of the media was supplemented with aphid homogenates. Aphid-supplemented minimal medium afforded the shortest LT_{50}s. There was no relationship between germination or appressorial development with LT_{50}s. The type of medium used for spore production influenced spore surfaces, primarily β-glucan content. There was a direct correlation between intensity of calcofluor fluorescence of stained conidia with conidial adhesion to host cuticles, suggesting this simple stain assay might be useful in determining potential virulence of conidia produced under various conditions. Measuring hydrophobicity of *Ma-an* conidia also has been suggested for screening fungal isolates with high propensity for attaching to insects (Jeffs and Khachatourians, 1997).

The ratios of dry rice and water introduced into bags before autoclaving vary with the producer and with the particular batch of rice being used. Parboiled rice with 100% (v/w) water and rice bran/husk with 70% water provided 6–7 $\times$ 10^9 *Ma-an* conidia g^{-1} substrate (Magalhaes and Frazao, 1996). Yield of *Ma-an* conidia from bran/husk medium in another study was 2 $\times$ 10^{10} spores g^{-1}, and this was calculated to be 80% of maximum theoretical production (Dorta *et al.*, 1996). The same count was obtained with wheat and rice grain, as well as three types of beans and sorgum (Allsopp *et al.*, 1994; Vilas Boas *et al.*, 1996). The mini-factory of LUBILOSA averaged 1.5 $\times$ 10^9 *Ma-ac* conidia g^{-1} of rice in 1997 (Jenkins *et al.*, 1998), and a Nicaraguan *Ma-an* production afforded 8 $\times$ 10^8 conidia g^{-1} of rice (Grimm, 2001).

The incubation vessels range from small bottles and plastic bags with as little as 100–400 gm. of rice to fairly large trays (approximately 0.5 m^2) (Alves and Pereira, 1998), and incubation is usually in a temperature-controlled room. The bags used normally are clear polypropylene. An inexpensive alternative to polypropolyene is the very thin polyethelyene bags widely used by supermarkets and department stores worldwide. These bags, unexpectedly, are fully autoclavable. The use of these bags for spore production was first seen by DWR in

Mexico (Raquel Alatorre, personal communication). They have been used routinely by the LUBLILOSA group in Cotonou, Benin, because they are both inexpensive and locally available. Bags are folded and stapled to insure sterility but still allow a small amount of gas exchange. Another approach to gas exchange is to close the bag with a cotton plug. Biocare Ltd. in Australia has a proprietary strip built into their plastic bags to insure oxygen and carbon dioxide exchange. In almost all cases, the bags with rice are autoclaved to ensure sterility. If a large autoclave is employed, there should be spacers placed between the layers of bags to insure sterility in a reasonable period of time.

Conidial suspensions are used as culture inoculum to initiate growth on the rice or other semisolid substrates in small production facilities. Inoculation is usually by repeating syringe with the needle inserted directly into the bag of autoclaved rice or other substrate. The bag may be wiped with alcohol before puncture and the hole closed afterward with tape. In large operations, the inoculum is mycelium in the log phase of growth produced in submerged culture. The mycelium initiates growth and sporulation considerably faster than inoculating with dormant conidia. In some cases, there is immediate massaging of the bag to evenly distribute the inoculum and to further ensure that the substrate is not aggregated. Incubation temperatures ranges from 25 to 30 °C, depending on temperature-control capability of the facility used and temperature optimum the strain of fungus. The most common temperature utilized worldwide seems to be 28 °C.

Another approach is to inoculate strips of nutrient-soaked absorbent paper or cloth with mycelium and incubate until spore production. Bailey and Rath (1994), tested 16 types of membranes and 9 nutrients. The most cost-effective nutrient was 2% skim milk powder and the best of the membranes was an absorbent fibrous material (Superwipe). Initial tests were promising, but cost efficiency of spore production decreased as the size of the membrane increased, and this approach was abandoned as less promising than the use of grain. The membrane system has been used for *B. bassiana* production by Nitto Denko Corporation in Osaka, Japan. Several factories in Brazil insist light (incandescent or fluorescent) in the incubation room is essential to adequate *Metarhizium* sporulation. Nevertheless, incubation of cultures in the dark normally affords high-levels of conidiation in the laboratory.

B. Spore Harvest

Harvesting of spores has taken several paths. The simplest is to use the fungus and its substrate for application directly into the field.

When rice or other grains are used they are similar to granulated formulations of chemical pesticides; moreover, they have the advantage of possible further sporulation after application. The Bio-Care Technology Pty. Ltd. (Australia) procedure calls for drying the spore-encrusted rice with vegetable-oil-coated 2-mm particles of bentonite, which reduces dusting and increases efficiency of application (Gary Bullard, personal communication).

A second, rather common harvesting method is to mill the fungus-infested rice grains and use the entire mix of spores and milled grain for field application. Care should be exercised in the milling process to prevent heat buildup, which can be lethal to the conidia. Milling, if done in an open environment, can cause unacceptable levels of dusting of the work environment with conidia.

The third approach is to separate conidia from the grain. One procedure used in Brazil was to float the spores from the grain by emersion in a large water bath. *Metarhizium* conidia are extremely hydrophobic and tend to float, while the rice tends to sink. A very efficient washing procedure was developed at a sugarcane cooperative, ASPLANA, near Maceio, Alagoas, Brazil, where the rice was passed in water between opposing spinning brushes to remove the spores (Messias, personal communication). The resulting spore suspension was then distributed to the grower for immediate application in the field. In this case, production of the spores was not initiated until the sugarcane plantation owner requested it, and the spores were delivered 16 to 18 days later. Interestingly, a second inoculation of the washed rice afforded higher spore yield than the first use. In low technology environments, spores washed from rice in mild detergent can be concentrated to a paste with a cream separator (Bailey and Rath, 1994). Kerosene also has been used to wash spores from rice (Lomer *et al.*, 1993; Magalhaes and Frazao, 1996). Metal sieves are widely used to separate conidia from rice: the rice is retained while spores fall through for collection. Sieve tables as large as 2 m^2 have been used in this process in Brazil. Again, this method can cause unacceptable levels of air contamination if proper venting is not utilized. In very large-scale production facilities, harvesting of spores usually employs some type of ''cyclone'' dust collector. These devices are in wide usage worldwide—for example, at flour mills, to separate small, light particles (e.g. conidia) from heavier ones (e.g. rice). The LUBILOSA group in Cotonou, Benin, used in their medium-scale production facility a clothes drier without heat to remove spores from rice, followed by cyclone harvesting and drying to the desired moisture in a simple drying chamber (Jenkins *et al.*, 1998). A spin-off of the LUBILOSA

harvesting equipment is a commercially available double-cyclone device (www.mycoharvester.info).

C. Storage of Spores

The rate of drying of the conidiated rice had some effect on survivability of conidia of a *Ma-ac* isolate (Hong *et al.*, 2000). Slow drying (room conditions at 25°, 45 to 75% RH for five days), reduced moisture content of the rice to about 30%. Rapid drying (forced-air drying cabinet at approximately 11% RH and about 20° for 16 hrs) reduced the initial 40% moisture content to about 20%. The conidia were further dried to 8% moisture content and sealed hermetically in laminated aluminum pouches. The test of survivability used a temperature of 50°C to accelerate the findings. There was a 71% increase in time to median lethality with the slow-dried conidia as compared to fast-dried.

A very important, but only recently recognized, factor impinging upon quality of the spores produced is that the water activity level of the substrate and alterations in medium to increase certain polyhydroxy alcohols (polyols) within spores will have serious impacts on their virulence and long-term survival (Magan, 2001). For example, *Ma* conidia produced on high glycerol medium adjusted to 0.96 a_w (water activity) contained elevated levels of glycerol and erythritol relative to spores from standard Sabouraud dextrose medium (SDA) (Hallsworth and Magan, 1994a). The former infected 70% of *Galleria* larvae at 78% equilibrium RH, whereas the latter failed to infect larvae at the same RH (Hallsworth and Magan, 1994b). The high-polyol spores germinate, albeit slowly, at equilibrium RH's as low as 89%, while control (SDA) spores required >99% RH (Hallsworth and Magan, 1995). An optimal set of nutritional and water activity conditions is suggested by Hallsworth and Magan (1996, 1999) for production of high quality *Ma* spores. Also, the duration of culture before harvest will affect survivability of conidia. There is an optimum time for collection, and variation from this time by only a few days can have a significant negative impact (Hong *et al.*, 2000).

Storage conditions are critically important if the spores are to be used in anything other than ''a fresh milk'' approach—viz., spores used almost immediately after production without longterm storage. With dried conidia, the key factors are temperature, oxygen content in the package, moisture content of the spores, and the packaging material. Because of cost, temperatures below freezing are seldom used for spore storage. It is well documented, however, that storage at refrigerator

temperatures (approximately 4 °C) affords much longer survival than higher temperatures; and that temperatures above normal room temperature can be severely debilitating. Where possible, oxygen in the package should be near zero (Miller, 1995; Roberts and Campbell, 1977), and RH below 8% (Daoust and Roberts, 1983a). Packaging is frequently in polyethylene bags. *Ma-an* conidia prepared by EcoScience Corp. (Jin *et al.*, 1999), and held at room temperature for two years and then at 4 °C for five more years had a germination rate of over 80% (Roberts, Rangel, Leite, and Braga, unpublished data). This system uses plastic-lined aluminum pouches with 0–10% relative humidity and nearly 0% oxygen [the package contains a desiccant (anhydrous calcium sulfate) and a pouch of oxygen scavenging material (powdered active iron oxide) used in the food industry (''Ageless,'' Mitsubishi International Inc.)]. The storage time of dry *Ma-ac* conidia has been extended from a few weeks in early experiments to four years at 10 °C or one year at 30 °C (Jenkins *et al.*, 1998; Lomer *et al.*, 2001). The dormant conidium has similarities to a plant seed, and seed technology concepts were found to be applicable to storing of *Ma-ac* conidia (Hong *et al.*, 1997, 1998, 1999, 2000). For example, there was a negative logarithmic relationship between longevity and conidial moisture content, with a lower limit of 4.6% moisture content below which conidial longevity did not increase. A model devised for predicting survival of *Beauveria* conidia in stored grain in Africa probably will be applicable also to *Metarhizium* in stored-product pest control (Hong *et al.*, 2002).

The preferred formulation for *Metarhizium* conidia for the control of many insect pests is various types of oil, ranging from vegetable oils to kerosene and mineral oil, as well as oil products designed by industry for pesticide formulation. Accordingly, storage in oil would be an advantage. Adding silica gel to the oil/spore mix (to dry the conidia) afforded survival (=80% germination) at 8 °C for 127 weeks and at 17 °C for about 51 weeks (Moore *et al.*, 1995). Predrying conidia to 15% moisture before adding oil without silica gel afforded survival at 25 °C for the full length of a four-month experiment (Hedgecock *et al.*, 1995). Adding a synthetic pyrethroid insecticide to conidia in oil maintained 90% germination for two months at 30 °C (Sanyang *et al.*, 2000), which would afford a short window between production and application in areas where refrigeration is not available. The mode of action was unknown and could be due to formulation compounds of the insecticide as well as to the insecticide. Formulation of *Ma-an* conidia in invert emulsion (oil-in-water) with a coconut/soybean oil preparation maintained 50% viability for 4.6 months at 20 °C;

whereas dry non-formulated conidia had a half-life of two weeks (Batta, 2003).

Although formulation with attapulgite clays afforded more than a year at room temperature of storage of *B. bassiana* conidia for (Feng *et al.*, 1994; Wraight and Carruthers, 1999; Wraight *et al.*, 2001), the same success was not obtained with *Ma-an* and *Ma-ac* using attapulgite clay or other clays (Daoust *et al.*, 1983; Moore and Higgins, 1997). Amorphous silicon dioxide, a sorptive dust, had a synergistic interaction when mixed with *B. bassiana* conidia and applied dry to stored-grain beetles (Lord, 2001). Rice or corn flour or phyllite formulation of *Ma-an* conidia allowed storage at room temperature for 9 months (Alves *et al.*, 1987). It should be noted that some of the above studies were done before the importance of drying of conidia to ~5% moisture was recognized.

Most *Metarhizium* strains, particularly those from grasshoppers, have greatly delayed germination following stresses such as long-term storage, heat, or UV exposure. This makes evaluation of survival difficult. Benlate added to the germination medium allows counting over several days because this fungicide kills fungi shortly after they produce germ tubes; and, therefore, a tangle of mycelium cannot develop (Milner *et al.*, 1991). Another approach is to use a surfactant that stimulates germination—for example, 0.05% ethoxylated tridecyl alcohol, with a hydrophile-lipophile balance (HLB) number of 10. Using dried *Ma-an* conidia, 24-hr. germination counts increased from 22% to 88% with the alcohol (Jin *et al.*, 1999). *Ma-ac* conidia dried to 5% water content had 25% germination when immersed directly into water; however, a 10-minute re-hydration in a moist environment prior to immersing increased germination to 77% (Moore *et al.*, 1997).

The recommended field dose for virtually all pests is 10^{12} to 10^{14} conidia per ha (Wraight and Carruthers, 1999; Wraight *et al.*, 2001). The LUBILOSA-recommended dose of *Ma-ac* is 10^{12} conidia per ha. Based on this relatively low dosage, the mini-factory in Benin in 1997 produced sufficient material to treat approximately 2000 ha. (Jenkins *et al.*, 1998). The cost of constructing a new *Ma-an* facility in Nicaragua with a capacity to treat 20,000 ha. annually was estimated to be US $260,000 and was expected to require outside funding assistance to escape local high interest rates (Grimm, 2001). Estimated treatment cost per ha is US $8–12. The need for products with high efficacy is apparent. This can take the form of: (a) application such that there will be recycling in the field after application; (b) monitoring to permit treating greatly reduced portions of the field (this is possible because most infestations of pests occur in hot spots instead of over an entire

area); (c) increasing the efficiency of production; and (d) finding, or producing through genetic or molecular-biology means, fungal isolates with increased virulence.

X. Formulation and Application

To formulate a microbial control organism is to mix ingredients to aid microorganism preservation, storage, application to the target, survival and activity (Burges and Jones, 1998c). The product of this activity is a ''formulation.'' The formulation may be designed for storage of the fungus and other ingredients added at the time of field application; or it may be packaged in field-ready form. In many pest-control situations, proper formulation is crucial to success. Failure to translate high mortality in the laboratory to the field environment often hinges on inadequacies in formulation and/or application coverage. The high importance of formulation and application are reflected in the number of reviews published recently (Bateman, 1997; Bateman and Alves, 2000; Bateman and Chapple, 2001; Bateman *et al.*, 2002; Batista-Filho *et al.*, 1998; Bernhard *et al.*, 1998; Burges, 1998b; Burges and Jones, 1998c; Inglis *et al.*, 2001; Jones, 1998; Jones and Burges, 1998; Moore and Caudwell, 1997; Wraight *et al.*, 2001). The comments above on storage of fungal products apply very directly to the subject of formulation. The planned application technology, of course, dictates many of the parameters of the final formulation. For example, applications of dust are very seldom done with entomopathogenic fungi, with the exception of control of stored-products (e.g., stored-grain) pests (Lord, 2001). Clays, however, are used as diluents and desiccants in shelf packs of conidia (e.g. Moore and Caudwell, 1997). The hydrophobic conidia of *Metarhizium* mix very well in oils, and oils of many types have been used as bases for fungal products. This approach has been particularly useful in dry environments, and success by LUBILOSA and others in low-humidity environments with oils has revolutionized thinking on the previously widely accepted concept that high humidity is required for fungal insect control. Oils have been used with chemical insecticides, so there is a considerable body of literature on oils appropriate to application to plants. These and other oils, such as kerosene or vegetable oils, were adopted by the LUBILOSA project to develop *Ma-ac* for locust control in the deserts of Africa. The scarcity of water in these areas necessitates spray applications in something other than water, and the researchers turned to ultra-low volume (ULV) applications conidia in oil at approximately one liter per ha. An Australian *Ma-ac* isolate afforded rather high infection levels with both

water and oil formulations at low RH in the laboratory (Fargues *et al.*, 1997). Although oils do not completely prevent wash-off from leaves by rain (Inyang *et al.*, 2000), a number of vegetable oils and commercially available formulations designed for insecticide application were found to more evenly spread *Metarhizium* formulations over leaves than water formulations (Ibrahim *et al.*, 1999; Malsam *et al.*, 2002). This spread is particularly important in the case of sessile insects such as immature whiteflies. An important caveat in using vegetable oils is that they can become rancid, and thereby produce compounds toxic to conidia (Inglis *et al.*, 2002). For this reason, commercially available oils referred to as paraffinic horticultural mineral and agricultural mineral oils are frequently used for formulating fungal spores. Methylated cottonseed oil has been used in North America and sunflower oil was effecitve in Europe (Malsam *et al.*, 2002). Oil-in-water or water-in-oil emulsions can be used to formulate fungal conidia (Batta, 2003; Inglis *et al.*, 2002), and these formulations can be applied with conventional application equipment. Phytotoxicity is low because of the low volume of oil; which, in fact, is usually less than 1% (v/v) (Bernhard *et al.*, 1998; Inglis *et al.*, 2002). Pure conidia or wettable powders can be excessively dusty and hydrophobic, requiring premixing with organosilicone wetting agents, e.g. Silwet (Osi Specialties, Inc.) or Sylgard (Dow Corning Corp.) (Wraight and Carruthers, 1999). Industry usually develops emulsifiable-oil formulations (ES = emulsifiable suspension), which obviate the dusting hazards and premixing complications.

XI. Epilogue

The current review focuses on mycological aspects of the genus *Metarhizium*. The genus includes several species, varieties within species, and individual isolates with broad ranges of physiological traits, including host range. The interactions of these fungi with their hosts, and the large literature on their use for pest control, largely define the scientific and popular concepts of *Metarhizium* spp. These topics will be addressed in a follow-on review in this journal.

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Molecular Biology of the *Burkholderia cepacia* Complex

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I. Introduction

The *Burkholderia cepacia* complex is a group of closely related bacteria found in diverse natural environments. They have been isolated from soil, water, plant, rhizosphere, and in animals including humans. They are pathogenic to both plant and animal and possess antibacterial and antifungal properties. Some members of the *B. cepacia* complex participate in the bioremediation of environmental pollutants while others are opportunistic pathogens that affect human health especially individuals with cystic fibrosis. In this article I would like to provide an overview of the molecular biology of the *B. cepacia* complex.

II. Characterization and Taxonomy

B. cepacia, a Gram-negative, aerobic nonfluorescent bacterium, was first described as a causative agent in the rotting of onion bulbs (Burkholder, 1950) and was originally classified as a member of the pseudomonads, thus the name *Pseudomonas cepacia*, in the γ-subclass of the *Proteobacteria* (Buchanan and Gibbons, 1974). It has been shown to catabolize a wide variety of organic compounds (Lessie and Gaffney,

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1986). Based on GC contents and rRNA/DNA hybridization studies, the pseudomonads were classified into five rRNA homology groups (Ballard *et al.*, 1970), and *P. cepacia* was assigned to rRNA homology group II (Palleroni *et al.*, 1973). Detailed sequencing analyses of the five rRNA similarity groups have confirmed the genetic distinctiveness of *P. cepacia* from the real pseudomonads such as *Pseudomonas aeruginosa* (Palleroni, 1992, 1993; Palleroni and Holmes, 1981). *P. cepacia* was subsequently reassigned to a new genus, *Burkholderia*, together with other members of the group II *β-Proteobacteria*, including *P. mallei, P. pseudomallei, P. caryophylli, P. gladioli, P. pickettii*, and *P. solanacearum* (Yabuuchi *et al.*, 1992). Further analyses based on 16S rRNA sequences have placed *B. cepacia* in a group together with *B. andropogonis, B. caryophylli*, and *B. gladioli* (Li *et al.*, 1993). The 16S rRNA of these bacteria exhibited 94% sequence similarity, which is significantly different from other members of the *Burkholderia*. The other members, including *B. solanacearum* and *B. pickettii*, that exhibited 88% homology were later reassigned to the genus *Ralstonia* (Yabuuchi *et al.*, 1995). In addition to 16S rRNA sequences, the 23S rRNA sequences (Ludwig *et al.*, 1995), the flagellin gene (Winstanley *et al.*, 2001) and the *recA* gene sequences (Eisen, 1995; Mahenthiralingam *et al.*, 2000a; Vermis *et al.*, 2002) have also been used for the classification of the *B. cepacia* complex. Comparison of the genomes by means of DNA macro-arrays, based on DNA-DNA reassociation, probably allows a more accurate and ultimate determination of the *Burkholderia* species (Ramisse *et al.*, 2003).

In spite of the various methods used for the classification of the *B. cepacia*, there are still difficulties in grouping of the bacteria, mainly because the bacteria have been found in a wide variety of environments that cover different ecological niches. The bacteria identified as *B. cepacia* are indeed a complex of closely related species or genomovars (Coenye *et al.*, 2001e; Ursing *et al.*, 1995). Since many *B. cepacia* strains were isolated and identified before the systematic taxonomic methods were available, accurate identification of the isolates as *B. cepacia* was almost impossible. The *B. cepacia* complex is thus a collection of *B. cepacia* and *B. cepacia*-like bacteria that include other *Burkholderia* (Coenye *et al.*, 2001b,d; Gillis *et al.*, 1995; Vandamme *et al.*, 1997, 2000), *Ralstonia* (Yabuuchi *et al.*, 1995), and *Pandoraea* (Coenye *et al.*, 2000a) species. The taxonomy of the *B. cepacia* complex has been reviewed by Coenye and coworkers (2001e). Figure 1 shows a phylogenetic tree of all the *Burkholderia* species based on 16S rRNA sequences available in the databases. It is obvious that the *B. cepacia* complex is composed of wide variety of species.

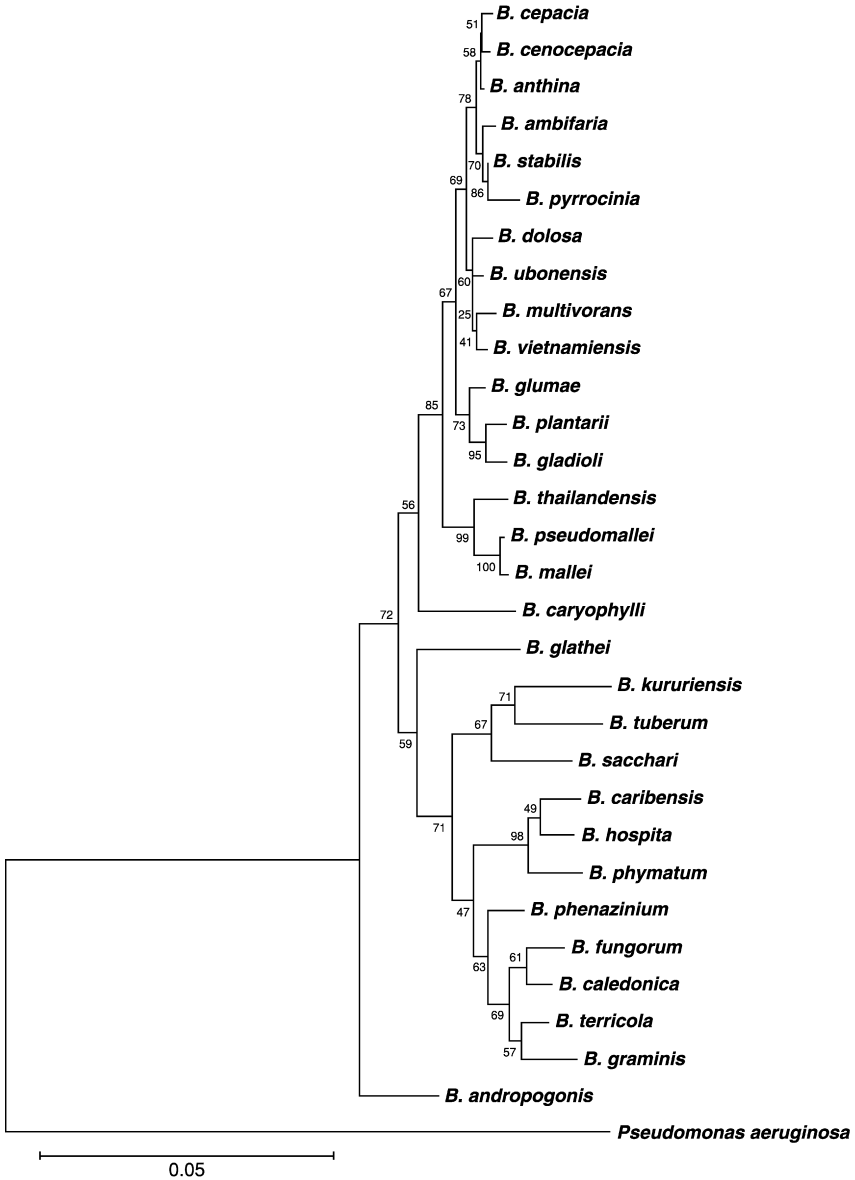

FIG. 1. A phylogenetic tree of all the *Burkholderia* species showing their relationships based on 16S rDNA sequence. The analysis was conducted by using MEGA version 2.1 (Kumar *et al.*, 2001). The tree was constructed using the neighbor-joining method with a bootstrap value of 500.

The *B. cepacia* complex has been shown to contain many species. The original isolate was identified as a plant pathogen (Burkholder, 1950). Other related *Burkholderia* species that are phytopathogens include *B. caryophylli* (Ballard *et al.*, 1970), *B. plantarii* (Azegami *et al.*, 1987), *B. glumae* (Goto and Ohata, 1956; Kurita and Tabei, 1967; Uematsu *et al.*, 1976), and *B. andropogonis* (Coenye *et al.*, 2001a; Smith, 1911). In contrast, species that exhibit anti-phytopathogenic activity, such as *B. gladioli* (Coenye *et al.*, 2000b), have also been identified (Attafuah and Bradbury, 1989; Burkhead *et al.*, 1994; Cartwright and Benson, 1995; Huang and Wong, 1998; McLoughlin *et al.*, 1992; Walker *et al.*, 1996). Most of the *Burkholderia* species are actually soil inhabitants. *B. caribensis* (Achouak *et al.*, 1999), *B. fungorum* (Coenye *et al.*, 2001c), *B. hospita* and *B. terricola* (Goris *et al.*, 2002), *B. gladioli* and *B. glathei* (Zolg and Ottow, 1975), *B. phenazinium* and *B. pyrrocinia* (Vandamme *et al.*, 2002b; Viallard *et al.*, 1998), *B. pseudomallei* (Brook *et al.*, 1997), *B. sacchari* (Brämer *et al.*, 2001), *B. thailandensis* (Brett *et al.*, 1998), and *B. ubonensis* (Yabuuchi *et al.*, 2000), are typical soil isolates. Others such as *B. graminis* (Viallard *et al.*, 1998), *B. caledonica* (Coenye *et al.*, 2001b), *B. dolosa* (Vermis *et al.*, 2003), *B. tuberum* and *B. phymatum* (Moulin *et al.*, 2001; Vandamme *et al.*, 2002a), *B. vietnamiensis* (Gillis *et al.*, 1995), and *B. kururiensis* (Zhang *et al.*, 2000) are isolates found in the rhizosphere that are able to fix atmospheric nitrogen and act as plant commensals (Estrada-De Los Santos *et al.*, 2001; Tran Van *et al.*, 2000). The species that attract the most attention are probably those that cause disease in animals, including humans. These include *B. ambifaria* (Coenye *et al.*, 2001d), *B. cenocepacia* (Vandamme *et al.*, 2003), *B. mallei* (Wheelis, 1998), *B. multivorans* (Vandamme *et al.*, 1997), and *B. stabilis* (Vandamme *et al.*, 2000). It is worth noting that many of the *Burkholderia* species have in fact been identified in multiple niches. For example, *B. multivorans, B. vietnamiensis,* and *B. ambifaria* have been found in both plant rhizospheres and in humans (Coenye *et al.*, 2001d; LiPuma *et al.*, 2001; Vandamme *et al.*, 1997). The distribution and probable ecological role of the *B. cepacia* complex in the natural environment have recently been reviewed by Coenye and Vandamme (2003).

III. Genomic Properties

The *B. cepacia* complex is unusual for its diverse ecological and functional properties. As noted above, the *B. cepacia* complex consists of bacteria isolated naturally from soil, water, plant rhizosphere, and animals. They have been used as antagonistic agents against plant

pathogens and as agents for bioremediation of environmental pollutants. On the other hand, they have also functioned as plant and animal pathogens. The ecological versatility of these bacteria is probably a result of their unusual genome organization.

A. Chromosome Multiplicity

By cutting the genomic DNA with restriction enzymes such as *Swa*I, *Pac*I, and *Pme*I that recognize rare sequences, and by resolving the DNA fragments with pulsed-field gel electrophoresis (PFGE), Cheng and Lessie (1994) showed that strain ATCC 17616 contains three circular chromosomes of 3.4, 2.5, and 0.8 megabases (Mb). This bacterium also contains a cryptic plasmid of 170-kb. Bacteria containing more than one circular chromosome have been confirmed (Allardet-Servent *et al.*, 1993; Trucksis *et al.*, 1998). Using a similar strategy and two-dimensional PFGE a physical map of strain ATCC 25416 has been constructed. This strain also contains three circular chromosomes but with sizes of 3.65, 3.17, and 1.07 Mb. A plasmid of 200-kb was also identified (Rodley *et al.*, 1995). A physical map for strain AC1100 (ATCC 53867), a laboratory strain that was selected on its ability to utilize 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), shows that it contains two chromosomes of 4.1 and 2.7 Mb and three plasmids of 500, 300, and 170-kb (Hendrickson *et al.*, 1996). By using PFGE and computational analysis of nucleotide sequences of genes available in the databases, the genome of *B. pseudomallei* strain K96243 has been estimated to contain two chromosomes of 3.5 and 3 Mb (Songsivilai and Dharakul, 2000). The complete nucleotide sequence of a type strain, *B. cenocepacia* (strain J2315), found in cystic fibrosis patients in U.K., revealed that it contains three chromosomes of 3.87, 3.22, and 0.88 Mb and a plasmid of 92.7-kb (http://www.sanger.ac.uk/Projects/B_cenocepacia/). A type strain of *B. fungorum* (LB400) that degrades environmental pollutants such as polychlorinated biphenyls (PCBs) contained three chromosomes and a plasmid totaling 8.1 Mb (http://genome.jgi-psf.org/draft_microbes/burfu/burfu.home.html). Analysis of the replicons present in 28 strains of clinical, environmental, or plant pathogenic origins show that strains within the *B. cepacia* complex contain two, three, or four chromosomes of larger than 500-kb and one or more plasmids. Clinical isolates tend to have more chromosomes than non-clinical ones (Wigley and Burton, 2000). The overall genome size of the *Burkholderia* isolates analyzed so far ranges from 4 to 9 Mb. This is roughly two times that of *Escherichia coli* and four times that

of *Haemophilus influenzae* (http://www.tigr.org/tigr-scripts/CMR2/CMRGenomes.spl).

B. Distribution and Organization of Genes

The presence of rRNA operon (*rrn*) genes in the replicon is an indication of a chromosome rather than a megaplasmid. Since *Ceu*I cut within the 23S rDNA and *Spe*I within the 16S rDNA sequences of *B. cepacia* (Dewhirst *et al.*, 1989; Hoepfl *et al.*, 1989; Liu and Sanderson, 1995), it is feasible to determine the number of *rrn* genes in the replicons by means of macrorestriction fragment analysis. The three chromosomes of ATCC 17616 have been shown to contain three, one, and one *rrn* operons, respectively (Cheng and Lessie, 1994). The number of *rrn* genes in the replicons was also confirmed by Southern blot analysis with 16S and 23S rDNA as the probe. Similarly, the three chromosomes of ATCC 25416 have been analyzed and were found to contain four, one, and one *rrn* operons (Rodley *et al.*, 1995). The AC1100 strain contains three and three *rrn* operons in its two chromosomes (Hendrickson *et al.*, 1996). Lessie *et al.* (1996) revealed that ATCC 29424 (Zylstra *et al.*, 1989), CRE-7 (Mueller *et al.*, 1996), and ATCC 17759 (McKenney *et al.*, 1995) all contain three chromosomes, and the first two contain six sets of *rrn* genes. ATCC 53617 contains four chromosomes with six sets of *rrn* genes. In summary, each of the chromosomes of *B. cepacia* contains at least one *rrn* operon.

The *recA* gene, which encodes for a multifunctional protein essential for DNA repair, homologous recombination and the SOS response, has also been analyzed in various isolates. By using the polymerase chain reaction (PCR) and specific primers Rodley *et al.* (1995) showed that each of the two largest chromosomes of ATCC 25416 contains a *recA* gene. A similar PCR analysis, in conjunction with Southern blot analysis, on 35 strains representative of the *B. cepacia* complex showed that only a single *recA* gene was found on the largest chromosome. Similar data were obtained from restriction fragment length polymorphism analysis (Mahenthiralingam *et al.*, 2000b). Since the *recA* genes of the *B. cepacia* complex have been shown to be rather heterogeneous (Chan *et al.*, 2003), further analysis of the location and copy number of the *recA* gene in the other strains still awaits further investigation.

In bacteria containing more than one chromosome, the essential genes were usually located in the largest replicon while non-essential and duplicated genes were normally found in the smaller chromosome (DelVecchio *et al.*, 2002; Salanoubat *et al.*, 2002). Genes required for the metabolism of 2,4,5-T have been cloned and used as hybridization

probes for studying the genomic organization of the 2,4,5-T degradative pathway in strain AC1100 (Hübner *et al.*, 1998). These genes were found distributed in the 53-kb and the 13-kb plasmids rather than in the chromosomes. Some of these genes existed as multiple copies and 2,4,5-T negative mutants occurred at a high frequency when the cells were grown in the absence of 2,4,5-T. These findings supported the notion that non-essential genes were usually located in the small replicons. The distribution and organization of the genes in ATCC 17616 have been analyzed by means of transposon mutagenesis (Rella *et al.*, 1985; Wong and McClelland, 1992). The largest chromosome contained genes responsible for biosynthesis of amino acids such as arginine, histidine, leucine, and threonine while the 2.5-Mb chromosome contained genes responsible for biosynthesis of lysine and β-lactamase as well as genes for the utilization of trehalose and phthalate (Cheng and Lessie, 1994).

Auxotrophic mutants of ATCC 17616 were also generated by means of transposon mutagenesis (Dennis and Zylstra, 1998). Most of these amino acid-requiring mutants were mapped to the largest chromosome. The histidine and the leucine biosynthetic genes were clustered in this chromosome while the tryptophan and the arginine biosynthetic genes were distributed between the two largest chromosomes. The lysine biosynthesis genes were located in the medium sized chromosome. Similar genomic organizations for these genes were also detected in *B. cenocepacia* and *B. fungorum* (Komatsu *et al.*, 2003). No insertion mutants were found on the smallest chromosome. Either the structure of this chromosome might be inhibitory to the maintenance or integration of the transposon or the essential genes were mainly located in this chromosome. Incidentally, this chromosome has many transposable elements (Cheng and Lessie, 1994).

All of the auxotrophic genes were also found to be single copy by allelic exchange mutagenesis, complementation, and Southern hybridization. This is in marked difference to that of the closely related *Ralstonia solanacearum*, in which duplicated or triplicated auxotrophic genes were detected (Salanoubat *et al.*, 2002). Multiple copy auxotrophic genes were also detected in other multichromosomal bacteria such as *Sinorhizobium meliloti* (Capela *et al.*, 2001) and *Agrobacterium tumefaciens* (Goodner *et al.*, 2001). This suggested that the genomic organization of the *B. cepacia* complex is different from the other multichromosome bacteria. While the auxotrophic genes among the members of the *B. cepacia* complex had similar organization, the *dnaK* and the *dnaA* genes flanking the *trpEGDC* genes of *B. cenocepacia* were located in different chromosomes in ATCC 17616 (Komatsu

et al., 2003). Suppression-subtractive hybridization (Diatchenko *et al.*, 1996) has been used to identify genes or genomic islands found in *B. cenocepacia* J2315 and not in non-transmissible genomovar IIIB strain. Many of the genomic regions exhibited abnormally low GC content and the distribution of three subtracted regions exhibited variations among members of the *B. cepacia* complex. A genomic island containing putative polysaccharide production genes was identified in some but not all genomovar IIIA strains. Two out of four genomovar I strains and one *B. multivorans* displayed this island while *B. vietnamiensis, B. stabilis, B. ambifaria,* and genomovar VI did not show these sequences (Parsons *et al.*, 2003). This indicated that there is still much genomic heterogeneity between individuals of the *B. cepacia* complex.

With the advancement of the sequencing techniques and the decrease in the cost of sequencing, it is now affordable to obtain the total sequence of a bacterium. Genomic sequences of *B. fungorum, B. cenocepacia,* and *B. pseudomallei* (http://www.sanger.ac.uk/Projects/B_pseudomallei/) are nearly finished. The sequencing of *B. mallei* is underway by The Institute of Genome Research (http://www.tigr.org/). For ongoing sequencing projects on the *Burkholderia* species, please visit the following website: http://wit.integratedgenomics.com/GOLD/. When complete genomic sequences of more members of the *B. cepacia* complex are known it will be possible to obtain a better picture of the genomic organization of these bacteria.

C. Insertion Sequences

Another property of the genome of the *B. cepacia* complex is its abundance of insertion sequences (IS). Insertion sequences are small (usually less than 2.5-kb) DNA elements that are capable of inserting at multiple sites in a target molecule. IS elements mediate gene mobilization, gene activation (Scordilis *et al.*, 1987; Wood *et al.*, 1990), replicon fusion (Barsomian and Lessie, 1986), gene rearrangement (Byrne and Lessie, 1994), as well as recruitment of genes (Lessie *et al.*, 1990). The *B. cepacia* complex harbors a large number of IS elements (Lessie and Gaffney, 1986; Miché *et al.*, 2001). Southern hybridization, using IS element-containing plasmids as the probe, has revealed that *B. multivorans* strain ATCC 17616 contains seven different IS elements. IS*403*, IS*406*, IS*407*, IS*408,* and IS*415* were detected in all three replicons while the smallest chromosome contains all seven IS elements including IS*401*, IS*402*. There are five, three, and one IS*403* found in the large, medium, and small sized chromosomes, respectively. Similarly, there

are two, one, and one IS*406* and IS*415*; one, two, and one IS*408*; one, one, and one IS*407* and zero, one, and one IS*401* (Cheng and Lessie, 1994). The 170-kb megaplasmid, pTGL1, of ATCC 17616 also harbors three IS elements, IS*405*, IS*408,* and IS*411*. The transposition of these IS elements promoted the formation of recombinant plasmids and generated a variety of genomic rearrangements (Gaffney and Lessie, 1987). The ability of AC1100 to utilize 2,4,5-T and the formation of deletion and rearrangement mutants have been strongly associated with the presence of these IS elements (Hübner *et al.*, 1998).

Ninety-nine strains of *B. cepacia* have been analyzed for IS*401*, IS*402*, IS*406*, IS*407*, and IS*408* by using PCR. Most of these strains were isolated from cystic fibrosis patients and the results revealed that 95% of the bacteria contain at least one IS element. Strains that were epidemic tend to contain more IS elements than strains of environmental origin (Tyler *et al.*, 1996). Specific analysis using dot blot hybridization for IS*1363*, a novel IS element found in epidemic strain PHDC that infects many cystic fibrosis patients in the mid-Atlantic region of the United States, was also investigated. Among 943 isolates, 761 from cystic fibrosis patients and the rest from the natural environment, IS*1363* was not found in other genomovar III strains and other *B. cepacia* complex species except *B. ambifaria* (Liu *et al.*, 2003). This supports the inference that the IS elements contribute to the genomic plasticity of the *B. cepacia* complex. The sizes of these IS elements range from slightly less than 1 kb to well over 3-kb. The characteristics of some of these IS elements have been reviewed by Lessie *et al.* (1996).

IV. Genetic Analysis Systems

A major constraint in the genetic study of the *B. cepacia* complex is that tools for its genetic manipulation and analysis are still at their developmental stages. Unlike pseudomonads, strains of the *B. cepacia* complex are normally resistant to many different types of antibiotics and the frequencies of transformation and conjugation are slightly low. Moreover, the existence of multiple replicons, the generally large genome size (>7 Mb), and the presence of large number of IS elements have made the complex rather heterogeneous and variable. Despite these inconveniences, genetic analysis systems that were originally developed for Gram-negative bacteria have been utilized for the study of the *B. cepacia* complex. In this section I will review some of the materials and methods used regularly for the genetic study of the *B. cepacia* complex.

A. Cloning Vectors

To understand the role of any of the cloned genes, it is necessary to analyze the gene of interest in the original organism. In general, narrow-host-range vectors regularly used in *E. coli* do not replicate in *Burkholderia*, but broad-host-range vectors specifically designed for the Gram-negative bacteria are usually functional. Most of the functional vectors used for *Burkholderia* are based on the replication origin of the RSF1010 (IncQ; Heffron *et al.*, 1975) and RK2 (IncP; Guiney and Yakobson, 1983). The RSF1010-based vectors are generally large because they have to contain three *rep* genes needed for replication and the origin of replication (*oriV*). Moreover, three *mob* genes and the origin of transfer (*oriT*) are also needed for conjugal transfer (Rothmel *et al.*, 1991). Recent constructs based on the RK2 are smaller and include an *oriV*, an *oriT,* and *parDE* genes required for plasmid stability (Blatny *et al.*, 1997; Scott *et al.*, 2003). Other general purpose shuttle vectors such as the pUCP family (Schweizer, 1991; West *et al.*, 1994) are based on the fusion of the origin of pRO1600 (*ori1600*) and the pUC18/19. Plasmid pRO1600 is an intermediate to high copy number cryptic plasmid isolated from *P. aeruginosa* that can replicate in many Gram-negative but not in *E. coli* (Olsen *et al.*, 1982). The pUC18/19 vectors are well-characterized vectors used in *E. coli* (Yanisch-Perron *et al.*, 1985). These vectors contain multiple cloning sites, appropriate antibiotic markers for direct screening, sites for sequencing and/or amplification, and a regulatable promoter for heterologous expression. These broad-host-range vectors are maintained in episomal condition and their copy number usually varies with the growth conditions. When the episomal condition is not favored then insertional vectors should be used.

B. Transposon Vectors

Transposons are complex mobile genetic elements that can be transferred from one species or replicon to another. They do not normally require any sequence homology for their integrative or transposition events and carry genes that mediate their own transposition. Most of the transposon vectors used in *Burkholderia* are insertional vectors based on the Tn*5* transposon. Tn*5* is one of the best characterized transposable elements and has been used extensively for many Gram-negative bacteria. Its site of integration is almost random in nature (Simon *et al.*, 1983). The Tn*5* variants were constructed to carry multiple cloning sites, and genes of interest could be cloned into the vector

and integrated into the host chromosome together with the transposition of the vector. This allows stable maintenance of the gene of interest even in the absence of selection.

To prevent secondary transposition and horizontal transfer, mini-Tn*5* derivatives are normally used nowadays (De Lorenzo *et al.*, 1990). These derivatives contain the two IS*50* terminal sequences, a unique rare restriction site such as *Not*I and an antibiotic resistance marker. They are placed in a suicide delivery plasmid that does not replicate in the recipient. The *tnp* gene that encodes the enzyme transposase, which is responsible for the transposition, is arranged in *cis* but outside the transposable element. Thus, when the vector is transferred and transposed to the recipient the integrated molecule will not be mobile anymore due to its lost of the transposase gene, a useful feature for clones targeted for environmental release.

Other derivatives of the transposon vectors contain a replication origin that is functional only in certain hosts. The vector can exist as a plasmid in certain permissive hosts, but only as an integrated transposon in the non-permissive hosts. Plasmid pOT182 contains the ColE1 replication origin and a Tn*5* derivative (Merriman and Lamont, 1993). Upon its mobilization into a non-permissive host such as *P. aeruginosa*, it can only exist as an integrated form. However, the plasmid can be released by cutting the genomic DNA, religated, and transformed into a permissive host. This manipulation allows genes adjacent to the integration site to be isolated and characterized. Similar self-cloning vectors have also been constructed specifically for *B. cepacia* (Abe *et al.*, 1996). A series of these self-cloning *plas*mid mini-trans*posons* have been named plasposons (Dennis and Zylstra, 1998). These plasposons contain the Tn*5* inverted repeats, a conditional replication origin, multiple cloning sites, and an antibiotic resistance cassette. The conditional replication origin could be an R6K replication origin that requires the π gene product to function properly (Kolter *et al.*, 1978) or it could be the replication origin of the narrow-host-range plasmid pSC101 (Cohen *et al.*, 1973). The modular antibiotic resistance cassettes include those for chloramphenicol, gentamicin, kanamycin, streptomycin, tetracycline, and trimethoprim.

C. Vector Delivery

While the first step in genetic analysis of cloned genes is the construction of utilizable vectors, it is still necessary to transfer the vector into the host cell. The transformation efficiency of the traditional method of treating the bacterial cells with divalent cation ions (Mercer

and Loutit, 1979) such as magnesium is far from optimal. Today most of the plasmids are transferred into *Burkholderia* by means of conjugation (Lanka and Wilkins, 1995) and electroporation (Dennis and Sokol, 1995).

Conjugation requires a special type of plasmid and cell-to-cell contact. Cloning vectors that contain the *mob* genes and *oriT* are transferable. For conjugal transfer membrane mating is regularly used in my laboratory. The donor and the recipient cells are cultivated separately in 2 ml of nutrient broth for 18 h at appropriate temperature. The cultures are then combined and filtered onto a sterile acetate cellulose filter (0.2 μm pore size), transferred to a nutrient agar plate, and incubated at 30°C for 24 hr. The cells on the filter are resuspended in minimal medium and appropriate dilutions are plated on selection plates that select against both parents. The genotypes of the transconjugants are then confirmed by appropriate methods (Provence and Curtiss III, 1994).

Electroporation is a process in which the bacterial recipient is subjected to a brief pulse of electricity. It is believed that the high voltage of electricity temporarily opens up the cell's membrane and allows macromolecules such as DNA to pass through. I have studied the various parameters that affect the electro-transformation efficiency of *Burkholderia*. While the electroporation medium is held constant containing 1 mM $MgCl_2$ and 1 mM HEPES, pH 7, the following parameters have been tested: the stage of the cells when they are harvested, the field-strength, and the quantity of DNA. Cells harvested at the late logarithmic phase give the best results while the optimal amount of DNA depended on the field strength applied. In general, 100 ng of DNA with a field-strength of 12.5 kV/cm gave the best efficiency. Up to 3×10^7 transformants per microgram plasmid DNA have been obtained (Tsang, unpublished).

V. Conclusion and Future Prospects

The taxonomy of the *Burkholderia cepacia* complex has advanced in the last 5 years. Molecular biology techniques have been used for accurate determination of species. Several genomes have been completely sequenced. The development of bioinformatics has also helped the processing of the large amount of information obtained from the various sequencing projects. With more and more hypothetical protein genes being identified in the *Burkholderia* species, systems for genetic analysis of the functions of these putative genes are needed. I have briefly reviewed some of the plasmid vectors and the genetic

analysis methods used for *Burkholderia*, but have not covered the use of bacteriophage that could possibly play a role in future investigations (Langley *et al.*, 2003; Matsumoto *et al.*, 1986; Nzula *et al.*, 2000). Transposon mutagenesis allows only the identification of non-essential genes. Future analysis with GAMBIT (*G*enomic *A*nalysis and *M*apping *B*y *In vitro T*ransposition; Akerley *et al.*, 1998; Wong and Mekalanos, 2000) will allow more systematic analysis of the genome including essential genes. The use of the enhanced green fluorescent protein gene (*e-gfp*) as reporter will simplify the analysis of the regulatory regions and facilitate the visualization and detection of *Burkholderia* in environmental samples (Lefebre and Valvano, 2002; Matthysse *et al.*, 1996). Moreover, with the availability of more genomic information of the *B. cepacia* complex it will soon be possible to monitor the abundance of the complex in the natural environment by means of metagenomic profiling (Sebat *et al.*, 2003).

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Non-Culturable Bacteria in Complex Commensal Populations

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I. The Commensal Microflora

All external surfaces of the human body are covered by microorganisms. This normal, resident or commensal flora is characteristic of the site at which it is found and generally lives in harmony with the host. This chapter will focus on the commensal microflora of the mouth and large intestine and examine its relationship with disease, the problems inherent in its study, molecular methods developed to investigate its unculturable component and how its evolution as a multi-species biofilm has led to the interdependence of its component organisms.

The commensal microflora protects the host from colonization by exogenous pathogens via the phenomenon known as colonization resistance (Vollaard and Clasener, 1994). The importance of this protective effect can be seen when the commensal flora is disrupted by, for example, antibiotic treatment (Sullivan *et al.*, 2001). In the mouth, this can lead to infection with the yeast *Candida albicans* and/or coliform organisms. In the gut, disruption of the normal microflora can lead to antibiotic-associated diarrhea or more rarely, pseudomembranous colitis, caused by infection with *Clostridium difficile* (Stoddart and Wilcox, 2002). The commensal oral microflora includes viruses, fungi, and protozoa as well as bacteria. *Herpes simplex*, the cause of cold sores, is frequently found in saliva (Youssef *et al.*, 2002) as is the yeast *Candida albicans*, the cause of thrush, but which is carried as a harmless commensal by approximately 50% of the population (Cannon and

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Chaffin, 1999). There are two species of protozoa found in the mouth: *Entamoeba gingivalis* and *Trichomonas tenax* (Wantland *et al.*, 1958). *E. gingivalis* is a simple amoeba and a close relative of those found in the environment, while *T. tenax* is a relative of the female genitourinary pathogen *Trichomonas vaginalis*. However, it is bacteria that dominate; saliva contains around 100 million bacteria per ml and includes representatives of more than 600 species (Kazor *et al.*, 2003). The large intestine harbours a similarly species-rich bacterial community with 10^{11}–10^{12} bacteria per gram of luminal contents (Guarner and Malagelada, 2003).

The commensal flora itself has normally been regarded as benign while diagnostic microbiology has focused on exogenous pathogens. However, increasingly, the ability of the commensal flora itself to cause disease is being recognised.

II. Diseases Associated with the Commensal Microflora

The oral microflora is perhaps unusual in that it has to be controlled to maintain health. It is unclear as to why at this stage of man's evolution the oral flora has to be controlled, but it has been suggested that the generally soft and carbohydrate-rich diet consumed in developed countries may be responsible. Certainly, the fermentation of dietary carbohydrate to produce acid is the principal mechanism in the pathogenesis of dental caries. The organism most commonly implicated in caries is *Streptococcus mutans* (Hamada and Slade, 1980), although there are a number of other acidogenic oral bacteria that can play a similar role (Kleinberg, 2002).

The periodontal diseases are a group of diseases affecting the gums. Dental plaque formation occurs as a bacterial succession whereby the initial colonisers are gram-positive aerobic bacteria. As the plaque biofilm develops it becomes successively more complex, more gram-negative and more anaerobic. Thus if the teeth are not cleaned at all or, more likely, when parts of the dentition are missed during an individual's normal brushing procedure, plaque accumulates on the teeth at the junction with the gums. Toxins, particularly endotoxin from the gram-negative species present, pass into the tissues causing irritation and inflammation, a condition known as gingivitis (Mergenhagen *et al.*, 1970). Gingivitis is thought of as a non-specific condition in that there are no specific organisms associated with its onset or progression; rather, the amount of plaque, and its maturity, correlate with disease severity (Ash *et al.*, 1964). It is reversible and the inflammation will subside if the plaque is subsequently removed (Loe *et al.*, 1965).

However, in some sites in susceptible individuals the lesions progress, so that attachment is lost between the gum and the tooth and a periodontal pocket is formed. The condition is then termed *periodontitis* and the pocket becomes heavily colonised with anaerobic bacteria. If left untreated, the pocket will continue to deepen, the supporting bone will recede away from the bacteria in the pocket, and eventually the tooth will be lost. The bacterial aetiology of periodontitis has been extensively studied and remains controversial. Although a wide variety of species are found at infected sites there is some evidence that disease severity is associated with only a restricted number of species, the so-called red cluster: *Porphyromonas gingivalis, Bacteroides forsythus* and *Treponema denticola* (Socransky *et al.*, 1998).

Both dental caries and periodontitis may also lead to abscesses either in the dento-alveolar area at the base of the tooth or in the surrounding soft tissues. Dental abscesses are always polymicrobial infections predominated by anaerobes, although milleri-group streptococci are also frequently isolated (Brooke *et al.*, 1991; Oguntebi *et al.*, 1982). Oral infections can have more serious consequences in that bacteria from oral lesions may travel through the bloodstream and cause abscesses of the liver, brain, or other internal organs (Crippin and Wang, 1992; Hollin *et al.*, 1967).

In the gut, members of the commensal flora are strongly suspected to play a role in the pathogenesis of the inflammatory bowel diseases ulcerative colitis and Crohn's disease. However, no specific organisms have been implicated, and it appears that it is an inappropriate immune response to the commensal microflora that is responsible for the conditions (Farrell and LaMont, 2002). Moreover, it has been suggested that some bacteria, such as lactobacilli and bifidobacteria, may be protective (Cummings *et al.*, 2003), and for this reason, probiotic approaches to the treatment of the inflammatory bowel diseases have been investigated (Isolauri *et al.*, 2002).

Traditionally these bacterial associations have been deduced from studies using conventional culture methods, which have clearly yielded much useful information regarding the diseases associated with the commensal microflora. However, bacterial culture is difficult and time consuming and in an attempt to provide greater accuracy and throughput, molecular methods such as DNA probing have been used to detect panels of organisms of interest. Although molecular probes are rapid and reliable, the choice of organisms for such studies was based on data available from culture. Culture studies suffer from the major deficiency that a significant proportion of bacteria making up the commensal microflora cannot be grown in the laboratory. In the

mouth, around only 50% of the bacteria are culturable (Wilson *et al.*, 1997), while it is estimated that 20–60%, depending on the study, of the colonic microflora is culturable (Guarner and Malagelada, 2003). Methods of studying ''unculturable'' bacteria are therefore clearly required.

In addition, traditional models of pathogenesis may not be appropriate for the understanding of the pathogenesis of diseases caused by the commensal flora. Mammals have co-evolved with their commensal flora; indeed 90% of the cells of the human body are bacterial. When one considers the large number of gram-negative cells in the human gut, with their constituent endotoxin, the lack of inflammation in healthy subjects is perhaps more surprising than the rarely encountered inflammation in those with inflammatory bowel diseases. It is increasingly recognised that the presence of a commensal flora is important for health and that human and bacterial cells communicate in a variety of ways to maintain homeostasis (Blum and Schiffnin, 2003). The diseases associated with the commensal microflora may, therefore, result from a failure of normal mechanisms rather than any aggressive behaviour on the part of the bacteria themselves. To study these mechanisms further, we clearly need to understand the composition of the complex bacterial communities that make up our commensal microflora.

III. The Problem of Unculturability

For the purposes of this chapter, the term *unculturable* is used to refer to organisms that are shown to be present in a sample by, for example, microscopic or molecular methods but which do not grow on conventional culture media under standard conditions. It is possible that they could grow if the conditions required for growth were reproduced in the laboratory. This is distinct from the phenomenon termed ''viable but nonculturable,'' which appears to be a survival mechanism adopted by some species under adverse conditions (Colwell, 2000).

Prior to the introduction of solid culture media, bacteria were grown in broth cultures. However, it is extremely difficult when using broths alone to be sure that the organism of interest is present in pure culture. For this reason Robert Koch recognized the benefit from growing bacteria on solid surfaces (Collard, 1976; Foster, 1970). To this end he used an aseptically divided potato as a medium for the growth of pathogenic bacteria isolated from human infections. By spreading the material over the surface of the potato he found that after a period of incubation, colonies of the bacteria grew which could then be transferred to other potatoes to obtain a pure culture. However, he quickly recognized, by

comparing microscopic and cultural counts, that not all, but perhaps the majority, of bacteria in the sample did not grow on the potato. This was perhaps the first recognition of the phenomenon of unculturability, where some of the bacteria in a sample are unable to grow *in vitro* under the conditions provided. Nevertheless, given the success of the use of solid media to obtain pure cultures of bacteria, this advance quickly led to the solidification of existing broth media with setting agents such as agar. Arguably, Koch's technique led to the golden age of medical microbiology during which time the majority of human pathogens were identified. Given this tremendous success, it is perhaps not surprising that an element of complacency developed and the substantial proportion of organisms that did not grow on the agar plates were overlooked. Put another way, it is extremely fortuitous that so many important pathogenic bacteria can be cultured in the laboratory, especially when we consider that on the earth as a whole less than 1% of the bacteria present can be cultured (Hugenholtz *et al.*, 1998).

IV. The Development of Methods to Study Unculturable Bacteria

It was first suggested by Zuckerkandl and Pauling (1965) that macromolecular sequence data could be used to elucidate evolutionary relationships between organisms. However, only with the advent of the modern techniques of molecular biology, particularly the polymerase chain reaction (PCR) and automated DNA sequencing, could the modern techniques of molecular ecology be used routinely. The methods are based on the comparison of sequences of genes that have been conserved throughout evolution. Genes encoding housekeeping functions are most commonly used as they tend to be widely distributed among the phylogenetic tree and, because of the need to preserve function, evolve slowly with time. The most widely used genes for this purpose to date have been that encoding the 16S ribosomal RNA (Olsen and Woese, 1993). At around 1500 base pairs in length, the 16S sequence is both informative enough to allow the identification of most organisms and short enough to be easily sequenced.

Comparison and analysis of 16S ribosomal RNA sequences allowed the construction of a new ''tree of life'' (Woese, 1987). Organisms could now be identified simply by placing their ribosomal RNA sequence on the tree. Because only the DNA was required, bacteria did not have to be cultured before they could be identified. In addition, by using cloning it was possible to separate mixed populations of genes so that complex bacterial communities could be characterized. These techniques were first applied to samples collected from the environment. For example,

Ward *et al.* (1990) examined the cyanobacterial mat at Octopus Spring by first isolating 16S rRNA from the mat biomass and then synthesising complementary cDNA, which was subsequently cloned and sequenced. Eight distinct sequence types were identified, all of which represented novel taxa. A different method was used by Giovannoni *et al.* (1990) to study bacterioplankton collected from the Sargasso Sea. DNA was extracted directly from the samples and the gene encoding 16S rRNA was amplified. The amplicons were then cloned and the 12 clones that were sequenced were found to belong to two novel lineages, related to the cyanobacteria and α-proteobacteria, respectively.

Following the demonstration of the potential of these methods for the characterisation of bacterial communities found in the environment, similar approaches were applied to the human associated microflora. Dymock *et al.* (1996) performed PCR/cloning analysis of the microflora associated with dentoalveolar abscesses and found that hitherto undescribed taxa were a major component. In addition to finding these novel taxa, it was also found that the numbers of certain well known species were severely underestimated by culture. These included *Porphyromonas endodontalis* and *Fusobacterium nucleatum*. Kroes *et al.* (1999) found that 52.5% of directly amplified 16S rDNA sequences from the gingival crevice represented novel taxa with 13.5% sufficiently divergent to represent new genera. Similarly, Paster *et al.* (2001) found that 215 of 347 taxa detected among 2,522 16S rDNA clones from subgingival plaque were novel phylotypes. Fourteen additional novel phylotypes were identified by Hutter *et al.* (2003) in a study of 26 subjects with advanced periodontitis. Kumar *et al.* (2003) used sequence data generated from earlier studies to design oligonucleotide probes to 42 oral taxa, culturable and non-culturable, and then applied them to samples collected from periodontitis subjects and controls. Associations with disease were found for several unculturable taxa including phylotypes D084 and BH017 from the *Deferribacteres* phylum, AU126 from the phylum *Bacteroidetes*, phylotype X112 from phylum OP11, and phylotype IO25 from phylum TM7. The two latter phyla have no cultivable representatives. Periodontitis-associated phylotypes described by other workers include PUS9.170 (Harper-Owen *et al.*, 1999) and AP24 (Sakamoto *et al.*, 2002). In a study of 5 patients with endodontic infections, Munson *et al.* (2002) found that 27 of 65 taxa identified were previously undescribed; 18 of these belonged to the phylum *Firmicutes*.

Molecular ecological studies of the faecal microflora have revealed similar findings. For example, Suau *et al.* (1999) found 82 taxa among 284 clones sequenced, 76% of which did not correspond to any known organisms. In an earlier study, Wilson and Blitchington (1996) made

the important observation that the number of cycles used in the PCR reaction influences the apparent diversity of the sample. From the same faecal sample, they found that 9 cycles revealed 27 distinct sequences among 50 clones while after 35 cycles, only 13 different sequences were seen among 39 clones.

V. Biases in Molecular Analysis

It is clear therefore that molecular ecological analysis reveals far more of the diversity of a bacterial community than culture. However, it is not free from its own biases. Each stage of the analysis has potential for bias (reviewed by von Wintzingerode *et al.*, 1997). First, the samples must be treated in such a way as to allow the equivalent recovery of DNA from Gram-positive and Gram-negative bacteria. Gram-positive cell walls tend to be thicker and more rigid than those of Gram-negatives. Most lysis protocols have been designed for Gram-positives with the assumption that procedures that lyse Gram-positive cells will also be suitable for Gram-negatives. Particular care should be taken with mechanical methods of cell lysis that can shear the DNA of those bacteria first to lyse (Leff *et al.*, 1995).

One fundamental source of bias is the number of ribosomal RNA operons on the chromosome. This number varies from 1 to 14 and thus the contribution of different taxa to the overall amount of 16S rDNA in a sample differs markedly and has been shown to affect the composition of the libraries obtained (Farrelly *et al.*, 1995).

The PCR stage is perhaps the step most susceptible to bias. First, from a theoretical standpoint, although the primers used for broad range PCR are described as universal, in fact, they have varying degrees of mismatch with organisms from different phyla. In addition, primer sets fail to amplify 16S rRNA genes from certain taxa for unknown reasons. One possible explanation for this phenomenon could relate to the G+C content of the DNA template. In addition to the relatively inefficient disassociation of high G+G templates, it is know that *Taq* polymerase may perform poorly with such templates, the enzyme pausing or prematurely terminating when it encounters G+C rich regions, owing to the formation of secondary structures (Henke *et al.*, 1997). The possibility that the composition of the DNA template is a cause of bias is supported by the observation that detection of members of the phylum *Actinobacteria*, the high G+C Gram-positives, is underestimated compared to culture in comparative studies (Munson *et al.*, 2002). A protocol to overcome this problem has been described by Baskaran *et al.* (1996), who suggested the addition of betaine and

DMSO to the amplification reaction and the use of two enzymes: Klentaq1 and Pfu DNA polymerase.

VI. Community Profiling

Molecular analysis via PCR, cloning, and sequencing, although extremely useful for the characterization of complex bacterial communities, remains time-consuming even with the introduction of automated DNA sequencers and robotic automation of DNA extraction and PCR procedures. As rapid alternatives, methods have been developed that give a profile of bacterial communities as a whole and, in particular, allow differences between communities to be identified. The most widely used of these has been the separation of amplified 16S rRNA gene PCR products by denaturing gel gradient electrophoresis or DGGE (Muyzer *et al.*, 1993). The technique is powerful because universal primers can be used to profile the entire community or group-specific primers can be used to focus on a lineage of interest. Once the amplicons have been separated, individual bands can be cut out of the gel and sequenced to identify individual taxa. Limitations of the technique are that amplicons from different taxa may migrate to the same position of the gel and the confounding effect of the formation of chimerae during the PCR. In such cases the polymerase ''jumps'' from one template to another forming a hybrid molecule derived from more than one organism. Nevertheless, DGGE and its variant, temperature gradient gel electrophoresis (TGGE [Rosenbaum and Riesner, 1987]), have proved extremely useful, particularly for identifying differences in the composition of communities. For example Zijnge *et al.* (2003) used DGGE to monitor the effects of treatment on the periodontal microflora and demonstrated that treatment, in the short term, reduced the diversity of the microflora and that the community that reestablished following treatment was significantly different to that present pretreatment. Fujimoto *et al.* (2003) also used DGGE to study the periodontal microflora and found that deep pockets harboured a more species-rich microflora than shallow pockets. Favier *et al.* (2002) used DGGE to follow the establishment of the intestinal microflora in faecal samples from two neonates up to 10 months. The initial simple microflora became more complex with time but was dominated by taxa belonging to or related to the genera *Bifidobacterium* and *Ruminococcus*. However, more than half the clones sequenced did not correspond to any known organism at the species level. A more focused examination of the diversity of faecal *Bifidobacterium* was performed by Satokari *et al.*

(2001) who used genus-specific PCR in conjunction with DGGE. They found that *Bifidobacterium adolescentis* was the predominant *Bifidobacterium* species in faeces in adults and that *Bifidobacterium* populations were stable over a 4-week period.

An alternative method for obtaining a profile of a bacterial community is that of T-RFLP (Liu *et al.*, 1997). PCR of a housekeeping gene (usually 16S rRNA) is performed with one of the primers fluorescent labelled. The amplicons are then digested with a restriction endonuclease and the fragments separated, typically using a capillary DNA analysis instrument. The terminal restriction fragment polymorphisms are therefore revealed as a profile representing all of the taxa in the community. T-RFLP has been used to explore the diversity of the periodontal microflora in patients with periodontitis and healthy controls (Sakamoto *et al.*, 2003). The profiles of disease-associated samples could be distinguished from the controls although there was substantial inter-sample variation. Individual subjects' profiles were stable over time.

VII. Detection of Unculturable Bacteria

Although of considerable academic interest, the demonstration of unculturable bacteria among the commensal microflora does not in itself further our understanding of diseases associated with the normal microflora. However, the studies described above have an important spin-off in that they generate DNA sequence data specific for the novel organisms present in the samples. From these sequences, specific oligonucleotide probes can be designed that can be used to detect the target organisms in samples by a variety of hybridisation methods including dot- or slot-blot hybridisation (Dix *et al.*, 1990) and real time PCR methods (Lyons *et al.*, 2000). They can also be used in conjunction with in-situ protocols to visualise the cells to reveal their morphology and with the use of confocal microscopy allow their spatial arrangement to be determined in biofilms (Amann *et al.*, 2001). They can also be used to label cells prior to analysis by flow cytometry and with cell-sorting techniques these bacteria can be physically isolated (Alvarez-Barrientos *et al.*, 2000). Once separation has been achieved, even if the cells are not viable, genomic DNA could be isolated from the organisms which could then probed for genes encoding virulence factors, antibiotic resistance determinants and enzymes typical of metabolic pathways that might give clues to the organism's growth requirements and eventually allow its *in-vitro* culture.

VIII. Why Are Some Bacteria Unculturable?

The reasons why some bacteria are unculturable have been sought for many years. Obvious reasons might be because the bacteria require a nutrient not present in the culture medium or that there is a toxic substance in the culture medium. When culturing mixtures of organisms from environmental or clinical samples, some bacteria may produce substances that inhibit others. Examples exist for each of these three possibilities. However, there may be other reasons for unculturability that stem from the biofilm habit of the commensal microflora. Almost without exception, the commensal microflora exists as a multispecies biofilm in which a wide variety of different taxa grow together in close proximity. Since these bacterial communities have co-evolved with their animal hosts over millions of years, it is possible that during that time some of the constituent species have become dependent on each other. For example, one species might have a mutation in an essential synthetic pathway but obtains the required substance from other bacteria in the biofilm. Only when the organisms are plated out to obtain pure cultures is the deficiency of the first species revealed. One example of this phenomenon would appear to be the oral organism *Tannerella forsythensis* (formerly *Bacteroides forsythus*). This organism grows extremely poorly in monoculture but grows well in coculture with other organisms such as *Fusobacterium nucleatum*. Wyss *et al*. (1989) showed that this near dependence was a consequence of *T. forsythensis* being unable to synthesise N-acetylmuramic acid (NAM), one of the essential components of cell wall peptidoglycan. Thus, *in vitro, T. forsythensis* can obtain NAM from other bacteria growing in close proximity. It also grows well in media supplemented with NAM.

In many ways bacterial biofilms resemble multicellular organisms (Stoodley *et al*., 2002). Organisms in a biofilm work together to share nutrients (Wei *et al*., 1999) and regulate their overall numbers by quorum sensing mechanisms (De Kievit *et al*., 2001). Bacteria also produce substances that regulate growth. For example, Mukamolova *et al*. (1998) described a protein produced by *Micrococcus luteus*, resuscitation-promoting factor (Rpf), which increased the viable cell count of dormant *M. luteus* cultures at least 100-fold at picomolar concentrations. Rpf also stimulates the growth of several other high G+C Gram-positive organisms including *Mycobacterium tuberculosis*. Rpf is a secreted protein that is essential for growth (Mukamolova *et al*., 2002). It would appear likely that more of these signalling factors regulating growth will be found and that there might be a network of

so called bacterial cytokines that regulate growth in bacterial communities. Some taxa may require such signals for growth, which would be a further explanation as to why so many organisms are unable to grow in monoculture *in vitro* when deprived of interaction with the other members of their normal biofilm community.

IX. Conclusions

Molecular analysis is revealing that a significant proportion (at least 50%) of the human commensal microflora is composed of as yet uncharacterized taxa. It appears that the evolution of bacterial communities in biofilm habitats may have promoted dependence on other organisms either via nutritional interactions or signalling networks. The understanding of diseases associated with the commensal microflora will first require the complete description of the bacterial communities present. The advent of the molecular techniques described here make this a feasible objective. However, because such diseases are likely to arise as a result of a complex interaction between the commensal organisms and the host, perhaps from a defect in homeostasis, novel techniques will be required for their investigation. These mechanisms are likely to have little similarity with the classical single organism, single disease interactions that were identified following the successful culture of bacteria in monoculture in the late 19th century.

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λ Red-Mediated Genetic Manipulation of Antibiotic-Producing *Streptomyces*

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I. Introduction

Streptomycetes are high G+C Gram-positive, antibiotic-producing, soil bacteria that undergo complex morphological differentiation (Chater, 2001). The 8.7 Mb *Streptomyces coelicolor* A3(2) linear chromosome sequence has recently been determined by using a series of overlapping inserts in cosmid vector Supercos1 that have proved very valuable in the technical developments that we describe in this article (Bentley *et al.*, 2002). The sequence analysis revealed 7,825 predicted open reading frames (ORFs), not including those of the liner 365 kb plasmid SCP1 and the 31 kb circular plasmid SCP2, which have been sequenced separately (Bentley *et al.*, 2004; Haug *et al.*, 2003). Recently the genome sequence of *Streptomyces avermitilis* has been published as well, comprising the 9 Mb chromosome and a 94 kb linear plasmid, SAP1 (Ikeda *et al.*, 2003; Omura *et al.*, 2001). Together

Abbreviations: $Carb^{R/S}$, carbenicillin-resistance/sensitivity; $Amp^{R/S}$, ampicillin-resistance/sensitivity; $Apra^{R/S}$, apramycin-resistance/sensitivity; $Cml^{R/S}$, chloramphenicol-resistance/sensitivity; $Kan^{R/S}$, kanamycin-resistance/sensitivity; *oriT*, origin of transfer

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these two genomes contain more than 50 gene clusters that are apparently concerned with secondary metabolites including antibiotics, siderophores, pigments, lipids, and other molecules with functions that are less well understood. The majority of these are present in only one of the two organisms. The further analysis of gene sets such as these, and of many other fascinating aspects of these complex bacteria, requires efficient tools for manipulation of their genomes.

Until recently, time-consuming restriction- and ligation-based techniques have been the principal route for introducing defined changes into the *Streptomyces* chromosome. Although useful, these methods are often inapplicable for large DNA fragments because suitable unique restriction sites are missing. This has engendered a growing demand for simple, one-step procedures for such purposes as gene disruptions, gene fusions, epitope tagging, or promoter replacements. Here we first describe the principles of some of the techniques recently developed by others. We then demonstrate how we have adapted and exploited these methods for *Streptomyces*, and provide some practical tips and discussion of common problems.

II. λ Red-Mediated Recombination in *E. coli*

In *Saccharomyces cerevisiae* and *Candida albicans*, the ability of linear DNA fragments to undergo recombination with the chromosome in the presence of homologies as short as 35–60 bp led to the development of PCR-based methods for gene replacement and modification, in which selectable antibiotic resistance genes are amplified by using PCR primers with 5′-ends homologous to sequences in the gene to be targeted (Baudin *et al.*, 1993; Wilson *et al.*, 1999). Unlike yeast, most bacteria are not readily transformable with linear DNA because the intracellular *recBCD* exonuclease (Exo V) degrades linear DNA. Efforts to circumvent this problem involved the use of mutants or conditions inhibiting Exo V (Dabert and Smith, 1997; El Karoui *et al.*, 1999; Figueroa-Bossi *et al.*, 2001; Russell *et al.*, 1989). However, these methods promoted homologous recombination with linear DNA only when the homology extension of the fragment exceeded a few hundred base pairs. More recently, it has been shown that expression of the *E. coli* RecE and RecT proteins or the corresponding recombination system of bacteriophage λ (the λ Red system) can greatly increase homologous recombination in *E. coli* (see reviews of Court *et al.*, 2002; Muyrers *et al.*, 2000; Poteete, 2001). Using this approach, it has been possible to achieve allelic replacement of genes located on the *E. coli* chromosome by PCR products containing ''homology arms'' of

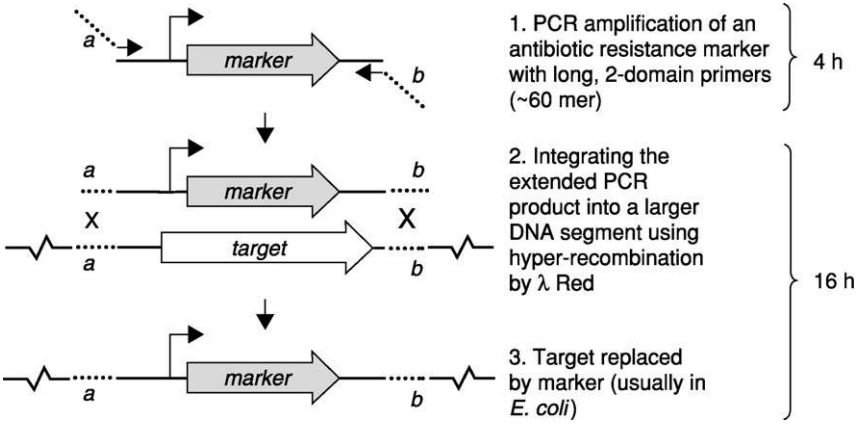


FIG. 1. Principle of λ Red-mediated replacement of a target gene with an antibiotic resistance marker. *a* and *b* (dotted lines) represent the 39 nt/bp extension sequences of the primer/PCR-product homologous to the adjacent sequence of the target gene. Starting with two gene-specific 59-nt primers, the entire procedure takes c.20 h.

36–50 bp, which form part of the commercially supplied customised oligonucleotide primers (Datsenko and Wanner, 2000; Murphy *et al.*, 2000; Yu *et al.*, 2000). Figure 1 shows a basic PCR-targeting strategy in *E. coli*.

III. Adaptation of Hyper-Recombination Systems for Other Organisms

Datsenko and Wanner (2000) demonstrated the use of λ Red recombination to disrupt 40 genes on the *E. coli* chromosome. Importantly, a set of disruption cassettes (templates for the PCR reaction) and plasmids for expressing the λ Red proteins were made available by depositing them at the *E. coli* Genetic Stock Center at Yale University (http://cgsc.biology.yale.edu). This has greatly facilitated the adaptation of this technology to other organisms by other scientists. In *Salmonella enterica* and *Salmonella typhimurium*, mutants have been generated by transforming λ Red-proficient cells with linear DNA fragments (Boddicker *et al.*, 2003; Bonifield and Hughes 2003; Boucrot *et al.*, 2003; Bunny *et al.*, 2002). A two-step strategy was used for the filamentous fungi *Aspergillus nidulans* and *Aspergillus fumigatus*: a λ Red-expressing *E. coli* strain containing a genomic region of interest (on a cosmid clone) was first targeted with a PCR-product to replace the gene of interest within the cosmid. The genetic exchange in the fungus itself was then achieved by homologous recombination between

the chromosomal locus and the recombinant cosmid after transformation with cosmid DNA (Chaveroche *et al.*, 2000; Langfelder *et al.*, 2002). The same strategy was used by Pérez-Pantoja *et al.* (2003) and Stewart and McCarter (2003) to generate deletion mutants in *Ralstonia eutropha* and *Vibrio parahaemolyticus*, respectively. All of these two-step approaches exploited the inability of the *E. coli* cloning vector to replicate in the target organisms: a feature that we have also exploited in the work on *Streptomyces* reported here. RecE/T mutagenesis was used to disrupt the ORF gG of bovine herpesvirus (BHV-1) cloned in a bacterial artificial chromosome (BAC) in *E. coli* (Trapp *et al.*, 2003). The mutant virus could then be recovered after transfection into bovine kidney cells.

These examples show that the technology is rapidly gaining widespread currency, and we anticipate that this will lead to the development of further novel applications that will be of wide general use.

IV. PCR-Targeting in *Streptomyces*

The strategy for PCR-targeting of *S. coelicolor* is to replace DNA in a sequenced *S. coelicolor* cosmid insert (Bentley *et al.*, 2002; Redenbach *et al.*, 1996) by a selectable marker that has been generated by PCR with primers with 39 nt homology arms (Gust *et al.*, 2003). In designing primers for PCR amplification, we took into account the observation from Yu *et al.* (2000) that λ Red-mediated recombination frequencies approach their maximum levels with a 40 bp targeting sequence. We chose to use 39 bp because it involves an integral number of codons, slightly simplifying the primer design without significant reduction in recombination frequencies. The inclusion of *oriT* (RK2) in the disruption cassette allows RP4-mediated intergeneric conjugation to be used to introduce the PCR-targeted cosmid DNA into *S. coelicolor* from *E. coli*. Conjugation is usually much more efficient than transformation of protoplasts and it is readily applicable to many actinomycetes (Matsushima *et al.*, 1994). The potent methyl-specific restriction of *S. coelicolor* is circumvented by mating the PCR-targeted cosmid from a methylation-deficient *E. coli* host such as ET12567 (MacNeil *et al.*, 1992). Vectors containing *oriT* (Pansegrau *et al.*, 1994) are mobilisable *in trans* in *E. coli* by the self-transmissible pUB307 (Flett *et al.*, 1997) or by pUZ8002, which lacks a *cis*-acting function for its own transfer (Paget *et al.*, 1999). For *Streptomyces* work, we constructed cassettes that can be selected in both *E. coli* and *Streptomyces* (Table I). The λ Red recombination plasmid pKD20 (Datsenko and Wanner, 2000) was modified by replacing the ampicillin resistance gene *bla* with

TABLE I

STRAINS AND PLASMIDS USED IN THIS STUDY

Strain/ plasmid	Relevant genotype/comments[a,b]	Source/reference
Plasmids		
pCP20	FLP-Recombination Plasmid: *flp, bla, cat, rep101*^{ts}	Cherepanov and Wackernagel, 1995
pIJ790	λ-RED (*gam, bet, exo*), *cat, araC, rep101*^{ts}	Gust *et al.*, 2003
pIJ773	P1-FRT-*oriT-aac(3)IV*-FRT-P2	Gust *et al.*, 2003
pIJ775	P1-(*Swa*I, I-*Sce*I)-*oriT-aac(3) IV*-(I-*Sce*I, *Swa*I)-P2	This study
pIJ776	P1-FRT-*oriT-neo*-FRT-P2	This study
pIJ777	P1-FRT-*neo*-FRT-P2	This study
pIJ778	P1-FRT-*oriT-aadA*-FRT-P2	Gust *et al.*, 2003
pIJ779	P1-FRT-*aadA*-FRT-P2	Gust *et al.*, 2003
pIJ780	P1-FRT-*oriT-vph*-FRT-P2	Gust *et al.*, 2003
pIJ781	P1-FRT-*vph*-FRT-P2	Gust *et al.*, 2003
pIJ784	bla-*oriT-aac(3)IV*-bla	This study
pIJ785	*ptipA*-P1-FRT-*oriT-aac(3)IV*-FRT-P2	This study
pIJ786	*egfp*-P1-FRT-*oriT-aac(3)IV*-FRT-P2	This study
pIJ787	bla-*oriT-tet-attP-int*-bla	This study
pIJ8641	*egfp, oriT, aac(3)IV*	Jongho Sun, personal communication
pUZ8002	*tra, neo*, RP4	Paget *et al.*, 1999
Supercos1	*neo, bla*	Stratagene
E. coli		
BW25113	K-12 derivative: Δ*araBAD*, Δ*rhaBAD*	Datsenko and Wanner, 2000
ET12567	*dam, dcm, hsdM, hsdS, hsdR, cat, tet*	MacNeil *et al.*, 1992
BT340	DH5α/pCP20	Cherepanov and Wackernagel, 1995
S. coelicolor		
M145	SCP1$^-$, SCP2$^-$	Bentley *et al.*, 2002

[a]P1, P2 left and right priming sites.

[b]bla represents 97 bp (left) and 301 bp (right) sequences matching left and right sequences of *bla* in Supercos1.

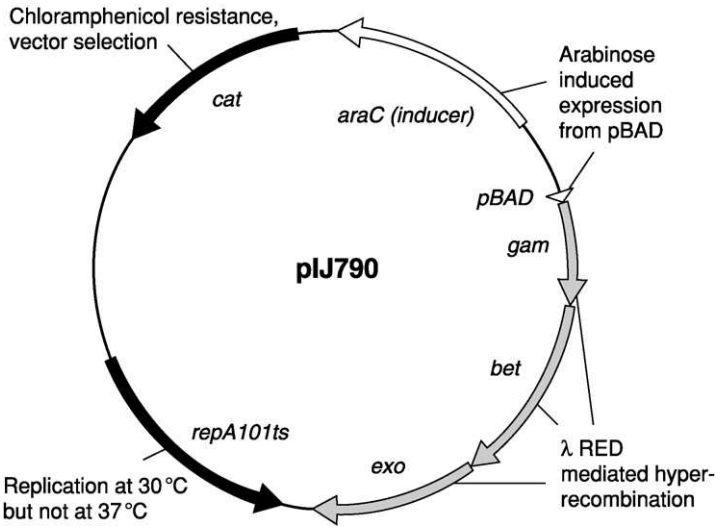


FIG. 2. λ Red recombination plasmid pIJ790.

the chloramphenicol resistance gene *cat*, generating pIJ790 (Fig. 2), to permit selection in the presence of Supercosl-derived cosmids (ampicillin and kanamycin resistant).

A. GENE DISRUPTIONS

A detailed protocol for generating gene disruptions in *Streptomyces* by λ Red-mediated PCR-targeting (Gust *et al.*, 2002) and a Perl Program (BMW) to assist in the primer design and in the analysis of the mutants generated are available at http://jic-bioinfo.bbsrc.ac.uk/S.coelicolor/redirect. Figure 3 shows the strategy for gene replacement in *Streptomyces*.

To demonstrate the high precision of the recombination occurring between 39 bp sequences, we initially chose to disrupt the *S. coelicolor* sporulation gene *whiI* (SCO6029) present in cosmid SC1C3. Many mutants defective in sporulation fail to produce the spore-specific grey pigment and appear white (Hopwood *et al.*, 1970), hence the designation of *whi* genes such as *whiI*. The *whiI* gene product is a response regulator-like protein (Aínsa *et al.*, 1999). Following transformation of the λ Red-expressing *E. coli* containing SC1C3 with a PCR-generated *whiI*-targeted $Apra^R$ disruption cassette, cosmid DNA samples of 50

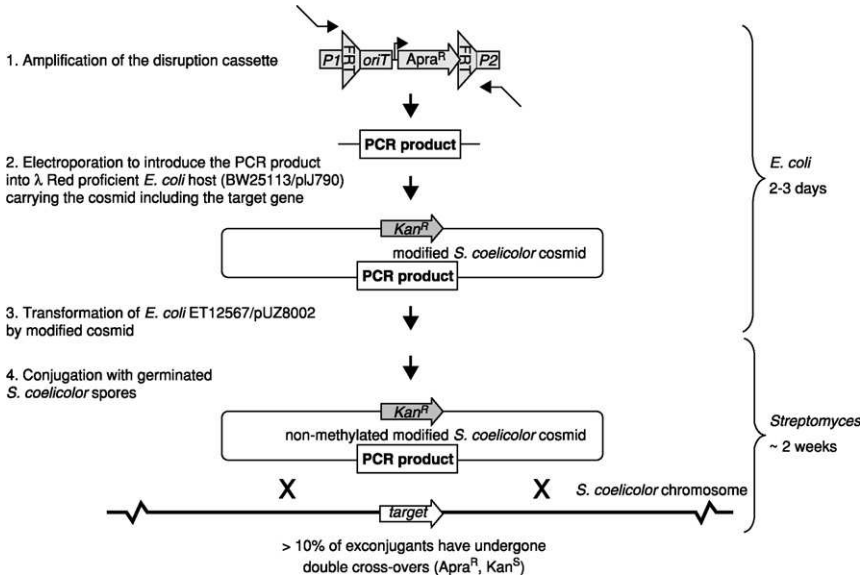


FIG. 3. Adaptation of PCR-targeting for *Streptomyces*. P1 and P2 represent the priming sites of the disruption cassette pIJ773. All disruption cassettes contain the same priming site for annealing of the long primers. FRT sites (FLP recognition targets) flanking the disruption cassette allow the elimination of the central part of the cassette to generate in-frame deletions (see below).

E. coli transformants were all shown to contain the desired gene replacement. The *whi*-disrupted cosmids were introduced into the methylation-deficient *E. coli* host ET12567 and mobilized into *S. coelicolor*. $Apra^R$ exconjugants that had lost the Kan^R marker of the cosmid were readily obtained. They were all white in appearance (Fig. 4), and for four independent mutants the gene replacement was confirmed by Southern blot and PCR analysis.

The technique has been used to disrupt more than 100 genes in *S. coelicolor* and has been successfully applied to different *Streptomyces* spp such as *S. spheroides* and *S. roseochromogenes* var. *oscitans* (Claessen *et al.*, 2003; Elliot *et al.*, 2003; Eustáquio *et al.*, 2003a,b; Gust *et al.*, 2003).

B. TIPS AND COMMON PROBLEMS

The most common problems we and others have encountered while using PCR-targeting in *Streptomyces* include the following:

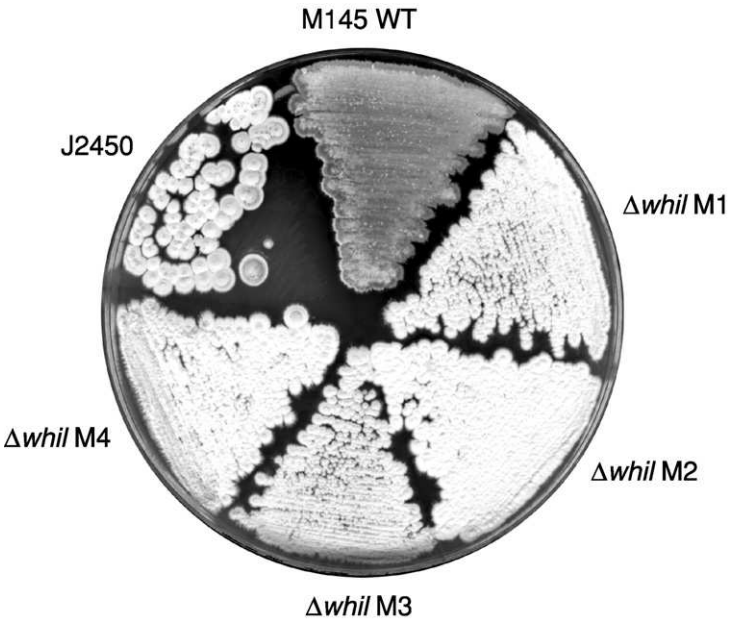


FIG. 4. Comparison of *whiI* mutants of *S. coelicolor* to the wildtype (M145) on MS agar. A previously, traditionally generated *whiI* null mutant, J2450 (Ainsa *et al.*, 1999) and four independent Δ*whiI* mutants (M1-M4), generated by PCR-targeting, all show the same white aerial mycelial phenotype.

1. *Little or no PCR-product is obtained*. The amount of template DNA is crucial for obtaining sufficient quantities of PCR-product for targeting. Approximately 100 ng of template should be used for the PCR reaction under the conditions given in the protocol (Gust *et al.*, 2002). Gene replacement was optimal with 200–300 ng of purified PCR-product.
2. *No transformants are obtained after PCR-targeting*. This common problem can mostly be resolved by using high-quality electrocompetent cells. It is important to start with the generation of competent cells immediately after the correct OD_{600} of 0.4–0.6 is reached without leaving the cultures on ice. Always keep the cells on ice between centrifugations. If no colonies are obtained after 16 h growth at 37 °C, repeat the experiment starting with a 50 ml SOB culture instead of 10 ml. Try to concentrate the cells as much as possible after the second washing step by removing all of the remaining 10% glycerol by using a pipette. Resuspend the pellet in the remaining drop of 10% glycerol (100–150 μl) and use this for electroporation.

3. *Different colony sizes are obtained after PCR-targeting.* After 12–16 hr growth at 37 °C different colony sizes are observed. It is important to note that, at this stage, wild-type and mutant cosmids co-exist within one cell, because, after transformation with a PCR product, not all copies in the cell will carry the disruption. One copy of a cosmid containing the incoming resistance marker is sufficient for resistance to the antibiotic, but nevertheless the larger the size of a colony, the higher the proportion of mutagenized cosmids. Cosmid copies lacking the disruption cassette will be lost during selection of the antibiotic resistance associated with the PCR cassette during subsequent transformation of the methylation-deficient *E. coli* host ET12567 containing the non-transmissible plasmid pUZ8002. This problem is not usually very important, because wild-type copies of the cosmid lack *oriT* and cannot be mobilised for conjugal transfer.
4. *Degradation of the isolated recombinant cosmid DNA*. This can easily be avoided by including a phenol/chloroform extraction step in the DNA isolation procedure even when using DNA isolation kits.
5. *The occasional presence of pseudo-resistant colonies on selective plates that fail to grow when transferred to liquid selective medium.* These can arise because of transient expression of the antibiotic resistance protein from the linear DNA (Muyrers *et al.*, 2000).
6. *No double cross-overs can be obtained in* Streptomyces. Typically, 5–70% of the exconjugants are double cross-over recombinants, if the gene of interest is not essential under the conditions of growth. The frequency of double cross-overs depends on the length of the flanking regions of homologous DNA on the cosmid. If <3 kb is present on one side of the disrupted gene, obtaining Kan^S double cross-over recombinants directly on the conjugation plates may be difficult. It may be necessary to streak out several exconjugants for single colonies or, more effectively, to harvest spores of Kan^R single cross-over recombinants and plate a series of dilutions on MS agar without antibiotics. After 3–5 days growth, the resulting colonies are replica-plated to nutrient agar with and without kanamycin, and screened for double cross-overs (Kan^S).

C. In-Frame Deletions

In the following two sections, we describe two different strategies to generate unmarked, non-polar in-frame deletions. The first includes site-specific recombination with the yeast FLP recombinase, which acts on FRT sites (*F*LP *r*ecognition *t*argets) flanking the disruption cassettes and allows removal of the antibiotic resistance and $oriT_{RK2}$.

The second strategy (''oligo-targeting'') takes advantage of co-transforming a λ Red-proficient *E. coli* host with oligonucleotides and linear DNA molecules.

1. *Using FLP Recombinase*

The plasmid pCP20 shows temperature-sensitive replication and thermoinducible expression of the FLP recombinase, which acts on FRT sites (Cherepanov and Wackernagel, 1995). FLP synthesis and loss of the plasmid pCP20 are induced at 42 °C. Expression of the FLP recombinase removes the central part of the FRT-flanked disruption cassette from the disrupted gene, leaving behind an 81 bp ''scar'' sequence, which lacks stop codons in the 27 codons that are present in the preferred reading frame (Datsenko and Wanner, 2000; Gust *et al.*, 2003). The resulting in-frame deletions are expected to be free of polar effects on downstream genes in operons. In addition, multiple gene disruptions can be generated by repeated use of the same resistance marker. Figure 5 summarises the procedure for obtaining such in-frame deletions.

2. *Using Oligo-Targeting*

For some purposes it maybe desirable to generate ''scarless'' deletions. The scar sequence contains some rare codons for *Streptomyces* and therefore could reduce the translation level of downstream, co-translated proteins. It also contains a functional FRT site that may interfere with subsequent rounds of deletions in the same cosmid. The disruption cassette-containing plasmid pIJ775 was constructed for this purpose. Like pIJ773, it consists of the apramycin resistance gene *aac(3)IV* and $oriT_{RK2}$. The cassette is flanked by two *Swa*I restriction sites (ATTTAAAT). The genome of *S. coelicolor* has only one natural *Swa*I recognition site (at position 1,821,751 bp), and there are no sites on the Supercos1 vector used for the ordered cosmid library. Replacing a gene on a cosmid with the pIJ775 cassette (Table I) will therefore allow the linearisation of the entire cosmid by restriction with *Swa*I. After agarose gel purification to eliminate uncut circular DNA, the linearized cosmid DNA can then be co-electroporated with a 80mer oligonucleotide, which consists of two 40 nt sequences homologous to the upstream and downstream regions of the target gene (i.e., containing the desired deletion junction), into a λ Red-proficient *E. coli* strain. Kan^R and Amp^R colonies only occur after recircularisation of the cosmid brought about by a double cross-over between the 5′- and the 3′-ends of the oligonucleotide and the ends of the linearized cosmid DNA (Fig. 6). In addition to the *Swa*I site, an 18 bp I-*Sce*I recognition site is included in the pIJ775 disruption cassette to allow

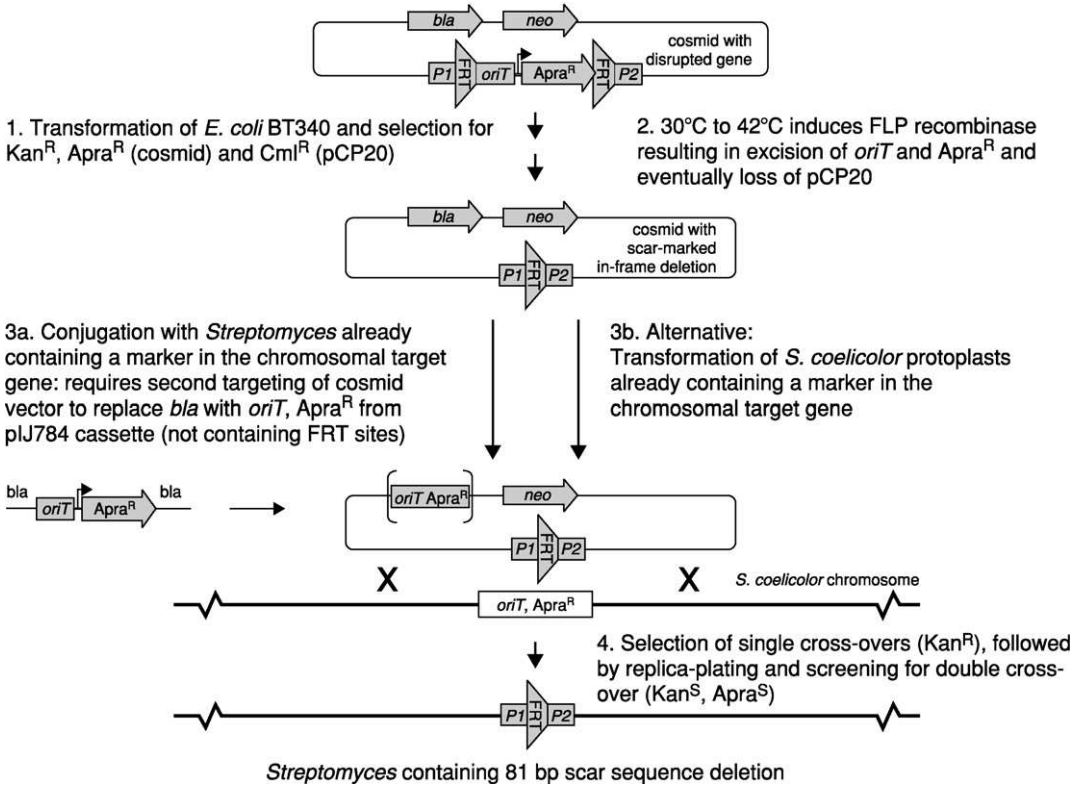


FIG. 5. Generating unmarked, in-frame deletions in *Streptomyces* by site-specific recombination with the yeast FLP recombinase. 3a and 3b are alternatives.

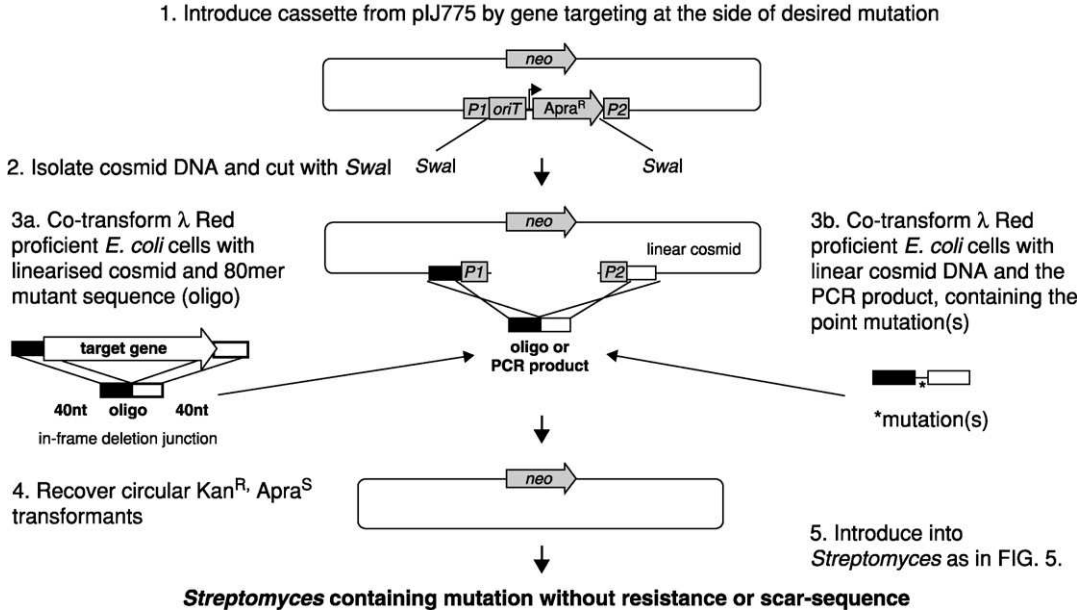


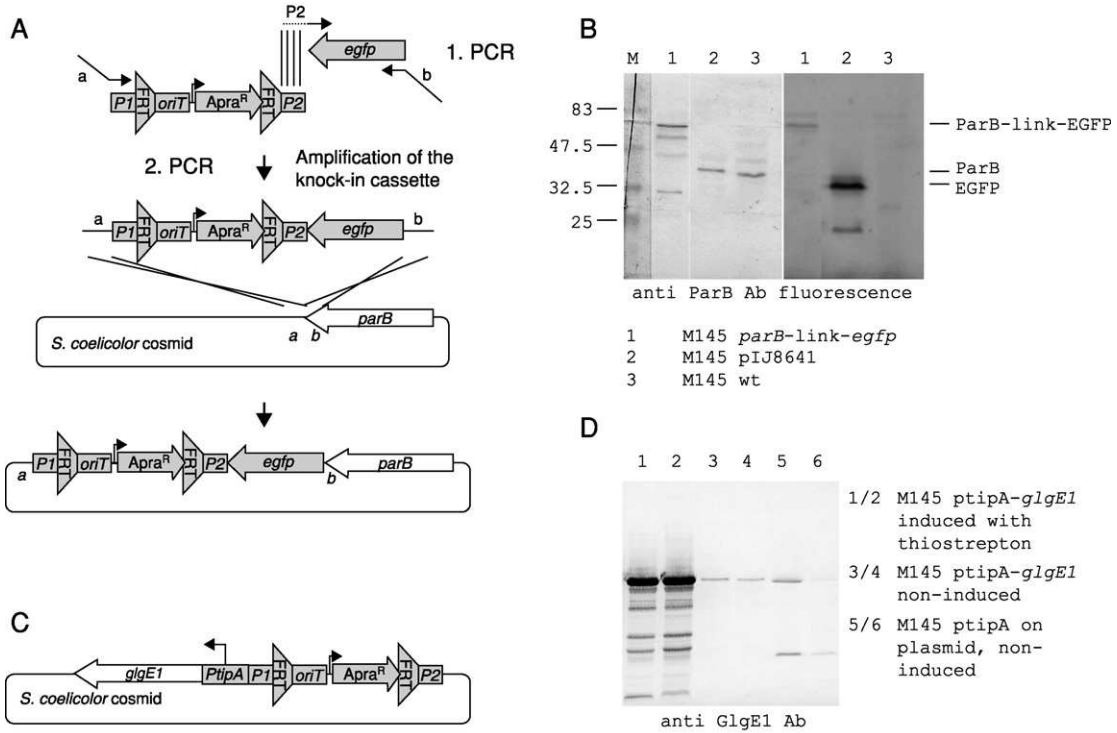
FIG. 6. Generating in-frame deletions or point mutations by co-transforming a λ Red-proficient *E. coli* strain with an 80-mer oligonucleotide (covering the deletion junction) or a PCR product (containing the point mutation) and linearized cosmid DNA. In the examples shown, *cyc2* deletion and *whiI* mutations were successfully introduced.

the generation of a scarless deletion *in vivo* by a double-strand repair mechanism. This complex strategy was used to engineer a reduced *E. coli* genome (Kolisnychenko *et al.*, 2002) and will not be further discussed in this work.

Oligo-targeting was tested with the *S. coelicolor* cosmid SC9B1 carrying the *cyc2* gene, which has been shown previously to be involved in the biosynthesis of the soil odour geosmin (Gust *et al.*, 2003). An 80mer oligonucleotide was ordered which would delete the entire *cyc2* gene after co-electroporation with the linearised, phenol-chloroform extracted cosmid DNA. Kan^R Amp^R transformants were screened by colony PCR with test primers annealing 100 bp upstream and downstream of *cyc2*. From 96 transformants, 25 were identified as positive candidates, whereas 37 seemed to show no deletion of the pIJ775 cassette insertion. This can be explained by incomplete *Swa*I digestion, resulting in still circular cosmid molecules which could transform the λ Red-proficient *E. coli* cells efficiently. Surprisingly, 34 transformants failed to produce any product in the PCR test. Restriction analysis of DNA of these 34 transformants showed deletions of different sizes on both sites of *cyc2*, which included the priming sites of the test primers. This could either be due to illegitimate intramolecular recombination of the linear cosmid DNA or to mis-annealing of the oligonucleotide at different positions within the cosmid insert. However, all 25 positive candidates were verified as scarless deletions of *cyc2* by restriction analysis and sequencing of the PCR product generated with the test-primers.

D. Introducing Point Mutations

It is valuable to be able to introduce point mutations into genes at their proper chromosomal location. It has been shown previously that RecE/RecT mutagenesis or λ Red recombination can be used to introduce point mutations at any position in a gene of interest (Muyrers *et al.*, 2000). Here we have used a simplified variation of this procedure to introduce point mutations into the *whiI* gene of *S. coelicolor*. In our version, λ Red recombination was not used to generate the point mutation itself, but instead it was used to introduce PCR fragments containing the point mutations into the corresponding cosmid by co-transformation as described for oligo-targeting (Fig. 6). Integrating different point mutations in the same gene requires only one initial gene replacement with the disruption cassette derived from pIJ775. In principle, any unmarked DNA fragment can be used for targeting as long as it is


A
P2
egfp
1. PCR
a
b
P1
FRT
oriT
ApraR
FRT
P2
2. PCR
Amplification of the knock-in cassette
S. coelicolor cosmid
parB
B
M 1 2 3 1 2 3
83
47.5
32.5
25
ParB-link-EGFP
ParB
EGFP
anti ParB Ab
fluorescence
1 M145 parB-link-egfp
2 M145 pIJ8641
3 M145 wt
C
glgE1
PtipA
S. coelicolor cosmid
D
1 2 3 4 5 6
1/2 M145 ptipA-glgE1 induced with thiostrepton
3/4 M145 ptipA-glgE1 non-induced
5/6 M145 ptipA on plasmid, non-induced
anti GlgE1 Ab

flanked by homologous sequences for λ Red recombination. Replacing chromosomal segments and module-swapping should therefore also be possible.

E. Promoter Replacements and Gene Fusions

Uzzau *et al.* (2001) have demonstrated the use of the Datsenko and Wanner procedure to introduce epitope tags into chromosomal genes in *Salmonella* by generating PCR templates containing a selectable marker and the epitope tag. Although this is straightforward and highly efficient, it relies on the construction of new template cassettes. Small tags like His-tags can be included directly into the long PCR-targeting primer sequence without the need to develop new cassettes. Here we describe how promoters can be replaced or gene fusions can be generated using the existing disruption cassettes.

The main idea is to use a two-step PCR, which in the first step generates a PCR product that contains the new promoter or the marker gene to be used for gene fusions. This PCR product is extended by choosing a primer sequence that allows annealing with the priming site of any of the disruption cassettes. For the second PCR reaction, the disruption cassette and first-round PCR-product serve as template and long PCR-targeting primers are used to amplify the complete ''knock-in'' cassette. The second-round PCR product can then be inserted directly at the desired position by λ Red-mediated recombination. Figure 7 shows two examples of such ''knock-in'' experiments, one a fusion of the enhanced green fluorescent protein gene *egfp* into the *parB* gene of *S. coelicolor* (Jakimowicz *et al.*, 2002) and the other a promoter replacement of the natural *glgEI* promoter by the thiostrepton-inducible promoter *ptipA* (Murakami *et al.*, 1989).

Fig. 7. Two examples of ''knock-in'' experiments. *A, egfp* is amplified in the first round PCR reaction to generate a PCR product flanked by sequences *b* and P2. The purified *egfp*-PCR fragment is then mixed with the disruption cassette and the knock-in cassette is generated by PCR amplification with the outside primers *a* and *b*. After λ Red-mediated targeting into the appropriate cosmid and introduction into *Streptomyces*, the ParB-EGFP fusion was analysed in *S. coelicolor* cell extracts using anti-ParB antibodies on Western blot and on the SDS-PAGE by phosphoimaging (*B*, left and right panels, respectively). *C*, Using a similar approach, the promoter of gene *glgE1* was replaced with the thiostrepton inducible promoter *ptipA*. Successful introduction of a functional construct into *Streptomyces* was confirmed by Western blot analysis of extracts prepared of cultures with and without induction by thiostrepton (*D*).

F. Heterologous Production of Antibiotics in *Streptomyces*

The genes for synthesis of any one antibiotic in streptomycetes are invariably clustered together on the chromosome (or sometimes on a plasmid). The availability of plasmid vectors which can efficiently carry stable large inserts into different *Streptomyces* spp. has been exploited in a number of laboratories to allow production in a heterologous host. Interspecies cloning of antibiotic biosynthesis genes or the corresponding resistance gene in non-producing hosts (often *S. lividans*) has been used to identify or confirm complete antibiotic gene clusters such as those for puromycin from *Streptomyces alboniger*, nikkomycin from *Streptomyces tendae* Tu901, kinamycin from *Streptomyces murayamaensis*, blasticidin S from *Streptomyces griseochromogenes*, complestatin from *Streptomyces lavendulae*, staurosporine from *Streptomyces sp.* TP-A0274, and rebeccamycin from *Saccharothrix aerocolonigenes* ATCC39243 (Bormann *et al.*, 1996; Chiu *et al.*, 2001; Cone *et al.*, 1998; Gould *et al.*, 1998; Lacalle *et al.*, 1992; Onaka *et al.*, 2002; Sanchez *et al.*, 2002). In addition, recombinant environmental libraries from soil for accessing microbial diversity were constructed as ''shotgun'' clones on an *E. coli—S. lividans* shuttle cosmid vector. New polyketide synthase genes were found with this approach in at least eight clones (Courtois *et al.*, 2003).

Many of the currently analysed antibiotic clusters are cloned on either cosmid or BAC vectors. Integrating these *E. coli* clones into *Streptomyces* chromosomes has the following advantages:

1. Introducing large DNA molecules into strains such as *S. coelicolor* or *S. lividans* has been established for a long time.
2. Using the methods described in this review, changes can be introduced into clusters within a few days and then rapidly integrated into the *Streptomyces* chromosome for further analysis.
3. Genetic tools have been widely established for some streptomycetes, making them desirable hosts for various genetic manipulations (Kieser *et al.*, 2000).
4. Once integrated, there is usually no need to maintain selection for the vector.
5. Where the clusters originate from streptomycetes or related high GC organisms, it is much more likely that they will express properly in a *Streptomyces* host than in *E. coli*.

To allow integration of any Supercos1-based cosmid into a *Streptomyces* chromosome, the targeting cassette in pIJ787 was constructed. It consists of a tetracycline resistance marker for selection in *E. coli* after

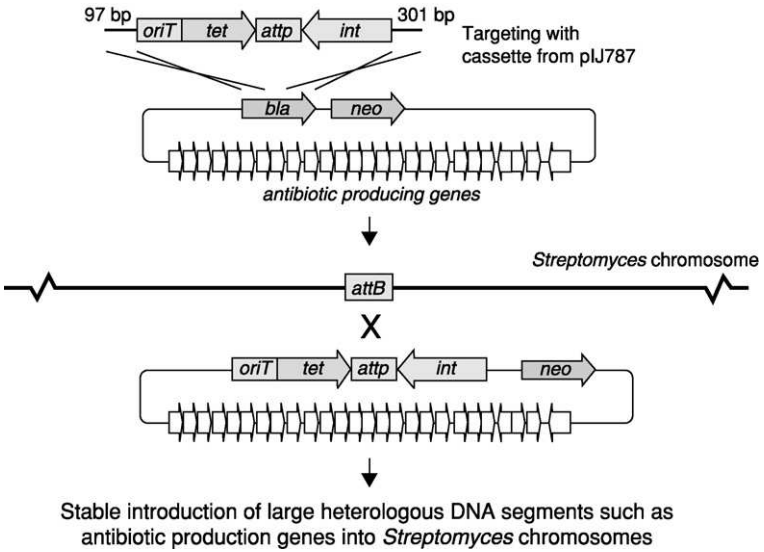


FIG. 8. Integrating cosmid clones into the chromosomal phiC31 attachment site present in *Streptomyces* species.

targeting, an $oriT_{RK2}$ for conjugal transfer and an *attP* site, and the integrase gene of phage phiC31 to allow integration into the chromosome by *attP*/*attB* recombination (Thorpe *et al.*, 2000). The cassette is flanked by sequences (97 bp at one end and 301 bp at the other end) homologous to the ampicillin resistance gene *bla* on the Supercos 1 vector backbone. This 4,990 bp cassette is cloned into Supercos1 and can be extracted as a *Dra*I/*Bsa*I restriction fragment, so that no PCR amplification is needed. The procedure to replace the ampicillin resistance gene *bla* on the cosmid vector by the plJ787 cassette (Fig. 8) is the same as described for gene disruption (Fig. 3).

As a test system, we used cosmid C73 of the ordered SCP1 cosmid library (Redenbach *et al.*, 1998), which contains the functional methylenomycin biosynthesis cluster (O'Rourke, 2003). Because methylenomycin is encoded by the linear *S. coelicolor* plasmid SCP1 (Wright and Hopwood, 1976), the recombinant cosmid C73_787 was introduced into the non-producing *S. coelicolor* M145 strain, which lacks SCP1. Successful integration into the M145 chromosome was confirmed with a bioassay for methylenomycin production (O'Rourke, 2003).

V. Conclusions and Future Prospects

We have adapted and exploited the use of λ Red recombination for use in *Streptomyces*. This rapid and highly efficient method has made the generation of gene disruptions more precise and allows the construction of in-frame deletions. So far, more than 100 segments of the *S. coelicolor* genome ranging in size between 4 bp and over 7 kb have been replaced by PCR-targeting. The technique has also succeeded in other *Streptomyces* species (Eustáquio *et al.*, 2003a,b). Here, we have described the use of this technology for various other DNA modifications such as introducing point mutations, promoter replacements, and gene fusions. Combining the different approaches enables us to manipulate *Streptomyces* DNA more rapidly and precisely than using traditional techniques. The facile integration of whole antibiotic gene clusters into *Streptomyces* chromosomes makes high-throughput manipulation of the clusters possible. Since it has been possible to reconstruct large gene segments (>90 kb) starting from pre-existing, smaller fragments by co-integrate formation and resolution *in vivo* (Sosio *et al.*, 2001) or by combining overlapping BACs with the help of λ Red recombination (Zhang and Huang 2003), even large antibiotic clusters will be manageable in the future. In combination with ET-cloning (Zhang *et al.*, 2000), swapping modules, for example within a polyketide biosynthetic megagene, should be achievable.

Acknowledgments

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Colicins and Microcins: The Next Generation Antimicrobials

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I. Introduction

The discovery of penicillin by Fleming in 1928 and the later purification and synthesis of this compound were historic milestones in the fight against infectious disease (Bennett and Chung, 2001). Subsequently, pharmaceutical companies discovered or developed numerous antibiotics effective against a wide range of human pathogens. Through a combination of administering antibiotics, improved sanitation and vaccination, rates of bacterial diseases rapidly fell. By 1975 the prevailing opinion was that the fight against infectious disease, at least in developed countries, had been won. Based on this trend, many pharmaceutical companies abandoned their antimicrobial drug development programs, as there seemed to be little need for new compounds (Knowles, 1997).

Antimicrobial resistance was observed shortly after the start of the antibiotic era in the early 1950s, but it was not until the 1990s that it became clear that the rate at which resistance developed was increasing rapidly (Levin *et al.*, 1998). In 2000 the World Health Organization

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cautioned that infectious disease may become untreatable because of the high levels of resistance of many human pathogens to the available drugs (World Health Organization, 2000).

The increasing threat of antibiotic resistance is largely the result of the overuse and misuse of antibiotics in human health as well as in agricultural and food production settings. Approximately one third of all hospitalized patients receive antibiotics and at least half of these prescriptions are unnecessary, poorly chosen, and/or incorrectly administered (Gaynes, 1997; van Houten *et al.*, 1998). In the agricultural industry the use of antibiotics for prophylactics and growth promotion has contributed significantly to the emergence of resistant bacteria in animals (Barton and Hart, 2001; van den Bogaard and Stobberingh, 1999). In addition, the almost exclusive reliance on broad-spectrum antibiotic agents is a contributing factor to the rapid emergence of multidrug resistant pathogens (Solomon *et al.*, 2001; Wester *et al.*, 2002). As novel mechanisms of antimicrobial resistance emerge in the pool of microbial pathogens, the frequencies of resistance have increased, although there is evidence that reduction in the use of broad spectrum antibiotics may result in improved microbial susceptibility (Melander *et al.*, 2000; Tan, 2003; Vlahovic-Palcevski *et al.*, 2001; Witte, 1998).

Recently there has been a renewed interest in finding alternatives to antibiotics. Agents such as bacteriophages (Alisky *et al.*, 1998), probiotic bacteria (Macfarlane and Cummings, 2002; Verschuere *et al.*, 2000), antimicrobial peptides (Joerger, 2003; Lowenthal *et al.*, 2000), bacteriocins (Joerger, 2003; Twomey *et al.*, 2002), and even phytotherapy (Shoskes, 2002) are under investigation. This review focuses on the bacteriocins produced by *E. coli* (known as *colicins* and *microcins*) and explores their potential role in agriculture, bioaugmentation, and human health.

II. Colicin and Microcin Characteristics and Functions

Colicins and microcins are the primary defense systems found in *Escherichia coli*. Well over 30 novel colicins have been characterized to date, and these high molecular weight proteins are found in about 30% to 50% of the isolates surveyed (Gordon *et al.*, 1998; Riley and Gordon, 1992; Smarda and Smajs, 1998). Microcins are much smaller than colicins and their synthesis, not lethal to the producing strain, is not SOS dependent (Pons *et al.*, 2002). Only nine microcins have been identified so far, and unlike colicins, very few of them are fully characterized at the level of structure or mode of action.

A. Colicins

Under conditions of stress, such as nutrient depletion or overcrowding, a small proportion of the potentially colicin-producing cells in an *E. coli* population are induced to produce colicins. Induction results in a suicide process whereby the producing cells actively participate in their own death while producing colicins that kill neighboring sensitive cells. The colicin proteins bind to specific cell surface receptors and are translocated into sensitive cells. As they enter the cell, they kill by one of several mechanisms (Table I): (i) channel formation in the cytoplasmic membrane, (ii) cellular DNA degradation, (iii) protein

TABLE I

Types of Colicins and Their Characteristics

Activity	Colicins	Receptor protein	Uptake system	Reference
Pore formation	E1	BtuB	TolCAQ	(James *et al.*, 1996)
	A	BtuB, OmpF	TolABQR	(Braun *et al.*, 1994)
	N	OmpF	OmpF, TolAQ	(Pugsley, 1984)
	U; S4	OmpF	TolABRQ	(Smajs *et al.*, 1997)
	K	Tsx	OmpFA, TolABRQ	(Pilsl and Braun, 1995)
	5; 10	Tsx	TolC, TonB, ExbBD	(Pilsl and Braun, 1995)
	Ia; Ib	Cir	TonB, ExbBD	(Braun *et al.*, 1994)
	B	FepA	TonB, ExbBD	(Braun *et al.*, 1994)
DNase	E2; E7; E8; E9	BtuB	TolABQR	(James *et al.*, 1996)
RNase rRNase	E3; E4; E6	BtuB	TolABQR	(James *et al.*, 1996)
	DF13	IutA	TolAQR	(Thomas and Valvano, 1993)
tRNase	E5	BtuB	TolABQR	(Masaki and Ogawa, 2002)
	D	FepA	TonB, ExbBD	(Masaki and Ogawa, 2002)
Inhibition of murine and lipopolysaccharide synthesis	M	FhuA	TonB, ExbBD	(Harkness and Braun, 1990)

biosynthesis inhibition by cleaving RNA, or (iv) murein and lipopolysaccharide biosynthesis inhibition by interfering with lipid carrier regeneration (Braun *et al.*, 1994; Riley and Wertz, 2002; Smarda and Smajs, 1998).

Despite these different modes of action, most colicin proteins share a similar organization, with three functional domains consisting of an N-terminal region involved in colicin translocation into the cell, a central region involved in colicin binding to specific outer membrane receptors, and a C-terminal region in which the killing activity resides (Braun *et al.*, 1994). The colicin receptor-binding domain binds to a specific cell surface receptor and is then taken up into the cytoplasmic membrane or the cytoplasm. Uptake through the outer membrane occurs via two different routes (Table I). One depends on cellular energy and requires the Ton system (ExbB, D, and TonB), while the other route involves the Tol system (TolA, B, Q, and R). Resistance to colicin killing can evolve through alterations in the cell surface receptors, or through changes in the uptake mechanisms (Braun *et al.*, 2002; Braun *et al.*, 1994; Riley and Wertz, 2002).

Colicins are encoded on either small multi-copy plasmids, which are amplified in the absence of protein synthesis and are not self-transmissible, or on large low-copy plasmids, which are not amplified and are transferred by conjugation. These plasmids contain colicin gene clusters usually composed of the colicin gene, which encodes the toxin; a constitutively expressed immunity gene, which encodes a protein that provides specific protection against the colicin; and a lysis gene, which encodes a protein involved in colicin release through lysis of the producer cell (Riley and Gordon, 1999; Riley and Wertz, 2002). Induction of the colicin gene cluster is controlled and regulated by the SOS response operon, which can be induced by DNA damaging agents and UV irradiation (Lu and Chak, 1996).

Colicins have provided a number of genetic and biochemical ''tools'' to microbiologists. Mechanisms of plasmid replication have largely been studied with colicin plasmids leading to the use of the ColE1 plasmid for the construction of the original pBR322 vector and its many derivatives (Bolivar *et al.*, 1977). Research on how colicins are translocated into the target cell and the mechanism of their export and import contributed to the development of colicin-based genetic tools that have been used in the study of protein transport across the envelope of Gram-negative bacteria (Filloux *et al.*, 2002). Colicins are released into the culture medium by a very different mechanism compared to other releasing systems found in *E. coli* and were shown to be applicable in mediating the release of a wide variety of heterogonous

proteins of different sizes (Dekker *et al.*, 1999; Lee *et al.*, 2001; van der Wal *et al.*, 1995).

B. Microcins

Microcins are antimicrobial peptides produced by and active against *E. coli* and its close relatives (Asensio and Perez-Diaz, 1976). Although they are similar to colicins in many ways, microcins can be distinguished from colicins by several key features: (i) size—colicins range in size from 25 to 80 kDa, while microbins are smaller than 10 kDa; (ii) secretion mechanism—microcins secrition, unlike colicins, is not lethal to the producing cell; (iii) induction—colicins are induced by the SOS system, whereas microcins are not; (iv) almost all colicins are plasmid encoded, while microcins are also found on the chromosome. Like colicins, microcin producers are immune to their own toxins (Kolter and Moreno, 1992). Microcin gene clusters are more variable in structure than colicin gene clusters, and include genes required for microcin biosynthesis (in some cases encoding a precursor peptide and posttranslational modification enzymes), and genes required for microcin secretion and immunity (Baquero and Moreno, 1984).

Microcins are actively secreted into the extracellular medium when cells approach stationary growth phase, except for microcin E492, which is mainly produced during the early log phase (Delorenzo, 1984). As is seen for colicins, microcin production is favored when nutrients are depleted and the cells become starved. Microcins are regulated by a number of stationary phase genes such as *rpoS, ompR* and *spoT* (Moreno *et al.*, 2002) as well as by nutrient deficiency agents such as the *fur* locus (Salomon and Farias, 1994).

Microcins share certain properties with the low molecular weight bacteriocins produced by Gram-positive bacteria, including thermostability, resistance to some proteases, relative hydrophobicity and resistance to extreme pH (Pons *et al.*, 2002). Pons and colleagues (2002) subdivided microcins into two groups. The first is composed of microcins B17, C7, J25, and D93, which undergo post-translational modifications, have a molecular mass of less than 5 kDa, and exert their antibiotic functions against specific intracellular targets. They inhibit vital functions of bacterial strains that share similar morphology and physiology (Table II). The second group includes E492, V, L, H47, and 24, which are larger peptides (between 8 and 10 kDa), are not post-translationally modified, are synthesized as precursor peptides and are mainly active through membrane potential disruption (Gaillard-Gendron *et al.*, 2000; Pons *et al.*, 2002).

TABLE II

TYPES OF MICROCINS AND THEIR CHARACTERISTIC

Activity	Microcins	Receptor protein	Uptake system	Reference
Pore formation and membrane potential disruption	E492	Cir, Fur,	TonB, ExbB, SmeA	(Braun *et al.*, 2002; Delorenzo, 1984)
	H47	Cir, FepA	TonB	(Patzer *et al.*, 2003)
	24	FhuA	TonB	(Braun *et al.*, 2002; Pons *et al.*, 2002)
Membrane potential disruption	V	FepA	TonB	(Braun *et al.*, 2002)
	L			(Gaillard-Gendron *et al.*, 2000)
Replication inhibition	B17	OmpF, OmpR	SmeA	(Destoumieux-Garzon *et al.*, 2002)
Membrane permeabilization	J25	FhuA	TonB	(Braun *et al.*, 2002; Salomon and Farias, 1992)
Translation inhibition	C7	Rep	OmpF	(Gonzalez-Pastor *et al.*, 1995)
DNA biosynthesis reduction	D93			(Martinez and Perezdiaz, 1986)

The antimicrobial properties of microcins and colicins (Tables I and II) make them excellent candidates for application in human and veterinary medicine (Table III). In this review, we will focus on the colicins and microcins that specifically kill human and animal pathogens thereby illustrating the potential of these compounds to serve as the next generation of antibiotics.

III. Potential Applications of Colicins and Microcins in the Livestock Industry

Swine, cattle, poultry, and other commercial livestock can be infected by numerous pathogenic enteric microbes, some of which are also infectious to humans. Virulent strains of *E. coli* (such as O157:H7) and *Salmonella enteritidis* of the serotype Typhimirium and related strains, cause intestinal and extraintestinal infection in humans as well as in animals and are the common microorganisms requiring antimicrobial therapy in livestock (Barton and Hart, 2001). *Salmonella* and *Campylobacter* account for over 90% of all reported cases of

TABLE III

PATENTED COLICINS AND MICROCINS APPLICATIONS

Number	Title	Application	Reference
5965128	Control of enterohemorrhagic *E. coli* O157:H7 in cattle by probiotic bacteria and specific strains of *E. coli*	Preventing the carriage of *E. coli* O157:H7 by a ruminate administrating an effective amount of colicin producing bacteria.	(Doyle *et al.*, 1999)
5549895	Method and colicin composition for inhibiting *Escherichia coli* O157:H7 in food products	Using colicin to inhibit the growth of pathogenic Enteriobacteriaceae in food and hard surfaces.	(Lyon *et al.*, 1996)
5043176	Biological control of food pathogens in livestock	Inhibiting growth of pathogenic Enterobacteria by using a *E. coli* host carrying microcin 24.	(Wooley and Shotts, 2000)
20030078207	Engineered antibiotic peptides and the preparation thereof	Engineer targeting antibiotic peptides using channel-forming colicin toxin domain and pheromone.	(Qiu, 2003)
20030039632	Novel bacteriocins transport and vector system and method of use thereof	Engineering lactic acid bacteria to produce gram-negative bacteriocins.	(Stiles *et al.*, 2003)

bacteria-related food poisoning worldwide (Trevejo *et al.*, 2003). Poultry and poultry products have been incriminated in the majority of traceable food-borne illnesses caused by these bacteria, although all domestic livestock are reservoirs of infection. *E. coli* O157 strains are also a concerning source of human infections. It was reported that up to 44% of cattle herds in Great Britain have at least one animal shedding *E. coli* O157 (Phillips, 1999).

To battle these common infections, livestock farmers rely on the use of traditional antibiotics such as penicillin, fluoroquinolone, and tetracycline. In addition, these drugs are used prophylactically and for growth promotion. As a result, high numbers of resistant strains have arisen not only in the pathogenic strains but also in the commensally enteric bacteria of the gut community (Barton and Hart, 2001; Mateu and Martin, 2001).

It is now recognized that it is critical to limit the use of traditional antibiotics to reduce the intensity of selection for resistance. To this

end, a number of strategies for the improvement of animal health, productivity, and microbial food safety that do not involve antibiotics have been explored. Dietary manipulation, competitive exclusion, and probiotics are under investigations to control and prevent enteric diseases in livestock (Phillips, 1999; Stevens *et al.*, 2002; van Immerseel *et al.*, 2002). In addition, bacteriocins (Cutter and Siragusa, 1995), bacteriophages (Alisky *et al.*, 1998), and antimicrobial peptides have been proposed as potential animal therapeutics (Joerger, 2003; Lowenthal *et al.*, 2000).

A. Colicins and Microcins in the Poultry Industry

Outbreaks of illness resulting from microbial contamination of chicken meat and eggs are a continuing source of economic and public health concern. *Salmonella enterica* serotype Typhimurium is a frequently isolated contaminant of poultry and is a commonly identified pathogen causing gastroenteritis in humans in the United States (Daniels *et al.*, 2002; Trevejo *et al.*, 2003). Methods used to inhibit, reduce, or eliminate *Salmonella* in poultry include the feeding of newborn chicks with ''normal'' chicken gut bacterial flora to inhibit colonization by the pathogenic strains of *Salmonella*, administration of avirulent *Salmonella* mutants to competitively prevent colonization by virulent strains, and use of food additives to alter the adhesion properties of pathogenic *Salmonella* (van Immerseel *et al.*, 2002).

Recent studies indicate that microcin producing *E. coli* strains can play a promising role in the prevention of *Salmonella* contamination in chickens (Portrait *et al.*, 1999; Wooley *et al.*, 1999). Wooley and Portarait both showed that in *in vitro* assays, microcin producing *E. coli* strains inhibit the growth of pathogenic *Salmonella. E. coli* can also survive and produce microcins in low nutrient media, under acidic conditions, and in the presence of bile and proteolitic enzymes; Wooley and his colleagues (2000) have further showed that strains engineered to produce microcin 24 can be used *in vivo* to inhibit the growth of *Salmonella* strains (Wooley and Shotts, 2000).

Microcins can also be useful indicators of pathogen presence in poultry. Certain strains of *E. coli* are associated with infections of extraintestinal tissues in chickens and in turkeys, ducks, and other birds. The initial infection usually occurs in the respiratory tract and results in a variety of avian diseases, including generalized infection, swollen head syndrome and cellulites, which are responsible for high levels of mortality as well as reduced growth, lower feed efficiency, and an increased condemnation rate at slaughter (Stehling *et al.*, 2003).

No clinical signs are associated with cellulites in living birds (Messier *et al.*, 1993); thus, easily identifiable markers of the virulent *E. coli* would be useful. Numerous reports have suggested that the majority (up to 92%) of virulent *E. coli* produce microcin V (Ngeleka *et al.*, 1996; Nolan *et al.*, 2003; Peighambari *et al.*, 1995). Moreover, introduction of microcin V plasmid significantly increases the virulence of the host bacteria. It was thus suggested that microcin V production and other virulence traits such as the presence of the increased serum survival genetic locus (*iss*) and complement resistance could be used to detect virulent avian *E. coli* strains. These proposed markers are currently under investigation (Gibbs *et al.*, 2003; Nolan *et al.*, 2003).

B. Colicins and Microcins in the Cattle Industry

E. coli O157:H7 is a shiga toxin producing strain that is the leading cause of haemorrhagic colitis and hemolytic uremic syndrome in humans (Phillips, 1999), the latter is characterized by lesions consisting of swelling and detachment of endothelial cells in the kidney. Hemolytic uremic syndrome can be fatal, especially in young children (Remuzzi and Ruggenenti, 1995). Cattle, chiefly young animals, are the major reservoir for *E. coli* O157:H7. According to the Center for Disease Control and Prevention (CDC), each year an estimated 73,000 cases of infection and 61 deaths in the United States are caused by *E. coli* O157:H7, mainly as a result of eating undercooked, contaminated ground beef (Phillips, 1999). In developing countries the frequency of infection and mortality is much higher.

The rumen appears to be the most important reservoir for *E. coli* O157:H7, and may serve as the source of the pathogenic bacteria found in the cow's colon. The traditional approach to protecting cattle from carriage of harmful bacteria is vaccination (Stevens *et al.*, 2002). Calves make O-antigen-specific antibodies in response to *E. coli* O157:H7 infection (Wray *et al.*, 2000), however, the induction of the antibody response is not correlated with elimination of the bacteria. Classical antibiotics have been employed as an alternative mode of therapy. However, the use of antibiotics to eliminate *E. coli* O157:H7 from the cow rumen is problematic, as several studies have shown that antibiotic therapy increases the amount of shiga toxin released and thus induces higher levels of bacterial virulence (Walterspiel *et al.*, 1992).

An alternative to antibiotics and vaccines to combat the proliferation of *E. coli* O157:H7 in the cow gut is the application of colicins and microcins, in probiotic and antibiotic formulations. These toxins are quite effective against the *E. coli* O157:H7 strain (Bradley *et al.*, 1991;

Murinda *et al.*, 1996; Sable *et al.*, 2000; Schamberger and Diez-Gonzalez, 2002), as well as other *E. coli* O serotypes associated with human diseases (Jordi *et al.*, 2001). Colicins E1, E4, E8, K, and S4 are the most effective growth inhibitors for *E. coli* O157:H7 under numerous environmental conditions (Jordi *et al.*, 2001). However, they induced colicin expression with mitomycin C (Pugsley, 1984) which, of course, is not present in the rumen and so the system still needs to be tested *in vivo*. Further, some *E. coli* strains isolated from the rumen can be used as probiotics, to reduce the frequency of *E. coli* O157:H7 in cattle and other ruminats (Doyle *et al.*, 1999; Stevens *et al.*, 2002; Zhao *et al.*, 1998). In summary, administration of probiotic, colicin producing bacteria can reduce the level of certain enteric pathogens in the cow gut and may also prevent animals from acquiring pathogenic strains.

IV. Potential Application of Colicins and Microcins as Food Preservatives

In recent years there has been an increased interest in using ''natural'' or biologically based methods of food preservation rather than chemical or physical preservatives. In particular, both bacteria and bacterial products have generated increased interest as preservatives. However, despite the wide range of potential microbial agents and products, relatively few of them have been developed for use in meat, egg, and dairy products. The only bacteriocins currently employed in food preservation are those produced by lactic acid bacteria (LAB). These fermenting bacteria have been used since ancient times for the preservation of meat and milk. More recently, nisin, a bacteriocin produced by *Lactococcus lactis subp. lactis* was approved for use by the FDA and has served as a model for the application of bacteriocins as food biopreservatives. Numerous studies have showed that LAB bacteriocins are safe for human use and have a huge potential as food preservatives (Cleveland *et al.*, 2001).

One drawback of LAB bacteriocins, however, is that they inhibit the growth of only Gram-positive bacteria (Rodgers, 2001). Gram-negative spoilage agents such as *Aeromonas, Escherichia, Salmonella, Yersenia* and *Pseudomonas* are rarely sensitive to LAB bacteriocins unless they are exposed to additional stress (Cutter and Siragusa, 1995; Helander *et al.*, 1997). To overcome this problem, microbiologists have attempted to create LAB bacteriocin derivatives that can specifically target Gram-negative bacteria. One example involves the use of microcin V, which is normally produced by the Gram-negative bacterium *E. coli*. The gene encoding microcin V was fused behind an LAB leader

peptide and the resulting construct was an LAB induced protein that had the activity of microcin V (McCormick *et al.*, 1999). These engineered LABs might be useful in preventing food poisoning caused by Gram-negative pathogenic bacteria in milk and meat products and could extend the shelf-life of these products (Stiles *et al.*, 2003).

Sable and colleagues (2000) identified microcin J25 as highly effective against *E. coli* O157:H7. The microcin was tested for activity in milk, egg, and meat extracts and was capable of inhibiting the growth of the *E. coli* O157:H7 strains present, suggesting that microcins might be used to control diarrheagenic *E. coli* strains in food products. Lyon and colleagues (1996) suggested spraying food products such as meats as well as the hard surfaces of meat processing equipment with powdered or liquid colicins. This approach has been successfully used with nisin reducing Gram-positive contaminations in fruit, meat, and surfaces (Barboza de Martinez *et al.*, 2002; Leverentz *et al.*, 2003).

V. The Potential Application of Colicins and Microcins for Environmental Remediation

A. Water Contamination

Waterborne bacterial contamination is a major concern in less-developed countries, where waterborne diseases are more common than in developed countries. Underground water currently supplies 30% of the world's population and is the main or only source of water for rural dwellers in many parts of the world. These water sources are often polluted by untreated sewage (Tibbetts, 2000). A recent survey suggests that it may be possible to treat water sources with bacteriocin-producing bacteria. In study of rural drinking water in Chile, it was shown that the bacteriocin-producing bacteria isolated from these water-sources inhibited the growth of *Samonella* and *E. coli* contaminations. It was thus suggested that bacteriocinogenic strains adapted to the natural conditions of the well sediment might decontaminate and control pathogens (Padilla *et al.*, 1990).

B. Bioaugmentation

Bioaugmentation involves the release of large quantities of microbial cultures, sometimes of recombinant species, to perform a specific remediation task in a given contaminated habitat. However, there is no way to predict the fate of the discharged bacteria because of the

variable conditions in the open environment (Wilson and Lindow, 1993). Various strategies have been developed to reduce transfer of new genes from the introduced organism to the local microbiota with gene and biological containment (Molin *et al.*, 1993). One such system is based on the lethality of colicin E3 RNase. The killing domain of the colicin E3 encoding gene was fused to the *lac* promoter and the construct was then inserted into the chromosomes of a variety of Gram-negative bacteria. Induction of the *lac* operon was lethal to the resulting bacterium in *in vitro* environments. This gene-containment mechanism provides neither an advantage nor a disadvantage to the host cell for survival, but may decrease the frequencies of productive chromosomal transfer in natural environments (Diaz *et al.*, 1994; Munthali *et al.*, 1996).

VI. The Potential Application of Colicins and Microcins as Antibiotics

The increasing prevalence of antibiotic-resistant pathogens has stimulated the efforts to identify novel antibiotics such as microcins and colicins. Research efforts in antibiotic development proceed along three tracks: (i) discovery oriented toward biological discovery of new agents from various sources; (ii) synthesis—chemical design of known and antimicrobials and their derivatives; and (iii) engineering—splicing together parts of the cell machinery from different clusters to build new potential drug. In this section we will provide an overview of novel uses and new antibiotics generated form colcins and microcins.

A. Colicin-Engineered Antibiotics

New approaches have been developed for fostering genes encoding traits from different organisms with colicins to engineer compounds with novel properties. One example of such a novel construct involves the fusion of a channel forming colicin Ia with a pheromone (*agrD*) produced by *Staphylococcus aureus* (Ji *et al.*, 1995; Mayville *et al.*, 1999). The fusion product (called pheromonicin) limited the growth of wild type *S. aureus* and two antibiotic resistant strains *in vitro*. Further, in mouse studies, the pheromonicin performed better than penicillin in eliminating the pathogenic *S. aureus* from the mouse. Similar studies with pheromones from *S. pneumoniae* and *Enterococcus facaelis* demonstrate that this pheromonicin is specific and fatal only to its intended victim (Qiu, 2003).

B. Colicin and Microcin-Induced Apoptosis

Apoptosis or programmed cell death is essential to all animal life. It is a key part in development and a safety feature in adulthood. The proper regulation and execution of apoptosis are essential to nearly all aspects of cell and organismal physiology, and deregulation of apoptosis contributes to diseases as diverse as cancer, autoimmunity, viral infection, and neurodegeneration. Some colicins and microcins toxins can induce apoptosis and some share common features with apoptosis-induced proteins (Jacobson *et al.*, 1997; Savill and Fadok, 2000). As a result they have the potential of becoming an attractive tool to understand apoptosis and might be utilized as cell targeted drugs. Some of the virulence factors of pathogenic bacteria can induce apoptosis (Weinrauch and Zychlinsky, 1999) and share common features with pore forming bacteriocins (Hetz *et al.*, 2002). For example, microcin E294 (isolated from *Klabsiella pneumoniae*) was shown to induce biochemical and morphological changes typical of apoptosis in human cell lines (Hetz *et al.*, 2002).

After apoptosis is activated, protein families (the caspase and the Bcl-2) participate in apoptosis as effector molecules (Weinrauch and Zychlinsky, 1999). Walker and his associates showed structural and mechanistic similarities between caspase responsible for DNA fragmentation and the active site of the cytotoxic endonuclease domain of colicin E9 (Walker *et al.*, 2002). It was also reported that the tertiary structure of the Bcl-2 family, which regulates apoptosis through permeability of cells organelles, resembles the toxin domain of pore forming colicins (Lazebnik, 2001; Weinrauch and Zychlinsky, 1999).

VII. Conclusions

Colicins and microcins were intensively studied for the past century. However, only recently has their enormous potential in agriculture, bioremediation, and human health have been recognized. This abundant and diverse family of toxins has the real potential of becoming the next generation of pharmaceuticals.

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Mannose-Binding Quinone Glycoside, MBQ: Potential Utility and Action Mechanism

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I. Introduction

Life-threatening fungal infections continue to increase in incidence for a variety of reasons, including advanced aggressive cancer chemotherapy, the use of highly effective immunosuppressants in organ transplantation, and the explosive increase in the incidence of human immunodeficiency virus (HIV) infection (Anaissie, 1992; Marr *et al.*, 1997). The emergence of azole-resistant isolates of pathogenic yeasts, particularly in HIV-positive and AIDS patients, as well as organ transplant patients, is a growing concern among infectious disease specialists (Heinic *et al.*, 1993). There is thus an urgent need for improved

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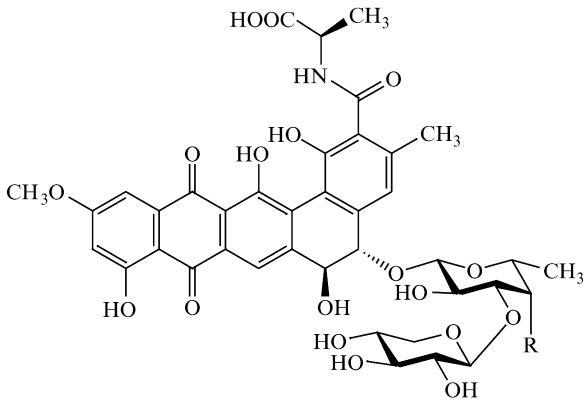


Pradimicin A R=NHCH$_3$
Benanomicin A R=OH

FIG. 1. Structure of MBQ.

antifungal agents that have potent fungicidal, broad-spectrum activity and safety superior to that of amphotericin B.

In the screening for microbial products as lead compounds for novel treatments for mycoses, pradimicin was found in the fermentation broth of *Actinomadura hibisca* (Oki *et al.*, 1988). Concurrently, benanomicin, an analog of pradimicin, was isolated from *Actinomadura* sp. by other group (Gomi *et al.*, 1988). Pradimicin and benanomicin, termed in this review MBQ (*M*annose-*B*inding *Q*uinone glycosides), are composed of a polyketide-derived benzo[*a*]naphthacenequinone aglycon, a D-amino acid and monosaccharide residues (Fig. 1). To date, 14 naturally occurring analogs and a number of synthetic derivatives have been reported with similar biological activities to pradimicin (Oki and Dairi, 1994). MBQ shows potent *in vitro* fungicidal activity and broad antifungal spectrum against a wide variety of fungi and yeasts, including clinically important pathogens and high *in vivo* therapeutic efficacy in the mice models against *Candida albicans, Cryptococcus neoformans*, and *Aspergillus fumigatus* (Kakushima *et al.*, 1991; Oki *et al.*, 1990).

MBQ recognizes D-mannosides as well as lectins, carbohydrate-binding proteins, and binds to the yeast cell surface in the presence of calcium ions (Sawada *et al.*, 1990). The binding is essential for MBQ to exert its fungicidal action, because the antifungal activity is antagonized by mannan, D-mannose, or a calcium-chelator EGTA (Ueki *et al.*,

1993a). Concanavalin A is a lectin that recognizes D-mannosides but does not show antifungal activity to yeast (Oki, unpublished result). The binding of MBQ causes a rapid leakage of potassium ions (Sawada *et al.*, 1990) and cytosolic small molecules such as nucleic acids and amino acids (Oki, unpublished result), and significant damages, especially in cellular and nuclear membranes in *C. albicans*. MBQ forms a water-insoluble complex with D-mannoside in the presence of calcium, which has a molar composition ratio of 2:4:1 (MBQ/D-mannose/calcium) (Ueki *et al.*, 1993a). MBQ strictly recognizes the orientation of the hydroxyl groups on a pyranose ring and the 2-, 3- and 4-hydroxyl groups of D-mannose are required for the stable complex formation (Ueki *et al.*, 1993b). Despite some efforts to understand the mechanism of complex formation, little is known about the molecular basis for the mannose recognition of MBQ (Fujikawa *et al.*, 1998; Hu *et al.*, 1999, 2000a,b; Ubukata *et al.*, 1997; Ueki *et al.*, 1993a,b,c).

In addition to antifungal activity, MBQ shows biologically important activities that are also, in principle, related to its binding ability to mannose-containing oligosaccharides. The HIV infection of T cells was inhibited by pradimicin A through the interaction with the high mannose-type oligosaccharides on the HIV envelope glycoprotein gp120 that promotes the viral fusion to T cell (Tanabe-Tochikura *et al.*, 1990). The syncytium formation of human parainfluenza virus-infected HeLa cells was enhanced by a pradimicin derivative BMY-28864 (Okamoto *et al.*, 1997). MBQ is a useful probe for detecting the expression of high mannose type oligosaccharides in a cellular event such as osteoclast formation from hematopoietic precursors (Kurachi *et al.*, 1994).

The present review will focus on the apoptosis of mammalian and yeast cells induced by MBQ and the possible molecular mechanism of fungicidal action.

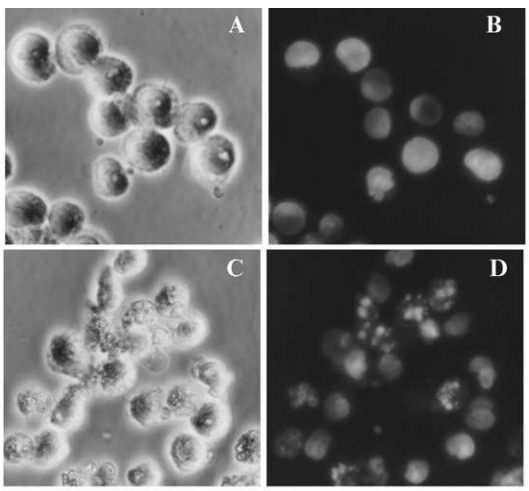
II. Apoptosis of Mammalian Cells Induced by MBQ

MBQ does not bind to mammalian cells because their cell surface oligosaccharides are modified with negatively charged sialic acids or neutral sugars such as *N*-acetylglucosamine; thus mannose residues are not exposed to the external cell surface. Accordingly, MBQ is not cytotoxic to cultured mammalian cells even at high concentrations (100$\sim$500 μg/ml) (Oki *et al.*, 1990). However, after the cultivation in the presence of 1-deoxymannojirimycin (DMJ), an α-mannosidase inhibitor, the cells express high levels of high mannose type oligosaccharides and become sensitive to MBQ (Ueki *et al.*, 1993b). In our early studies using yeast, specific binding sites were hardly detectable,

mainly because of the non-specific binding to the abundant cell surface mannan. To minimize this background, a human myeloid leukemia U937 cell was used for the mechanistic study, hypothesizing that a mammalian cell expressing high mannose type oligosaccharides would respond to MBQ in a similar manner as yeast cell.

A. Cell Surface Oligosaccharide-Dependent Apoptosis

As expected, MBQ showed cytotoxic effects on U937 cells pretreated with DMJ but not on untreated cells. The effect was dependent on the binding of MBQ to the cell surface as observed in *C. albicans* (Oki *et al.*, 1997, 1999a). Similar results were obtained with HL-60 and PC12 cells, implicating a common mechanism of MBQ-induced death in cultured mammalian cells. Interestingly, after exposure to MBQ, the DMJ-preincubated U937 cells showed characteristic apoptotic morphology (Fig. 2, see color insert). The entire cell and nucleus budded into small spherical structures called *apoptotic bodies*, and the condensation and fragmentation of nucleosomal DNA was detected by Hoechst 33258 staining and DNA ladder formation after electrophoresis on an



Cells were treated with 20 μg/ml MBQ for 24 h.
A/B: control cells. C/D: cells preincubated with 200 μg/ml DMJ for 48 h.
A and C: Nomarski optics. B and D: Hoechst 33258 staining.

Fig. 2. Apoptosis induction by MBQ in U937 cells preincubated with DMJ. Cells were treated with 20 μg/ml MBQ for 24 hr. *A/B*, Control cells. *C/D*, Cells preincubated with DMJ for 48 hr. *A* and *C*, Normarski optics. *B* and *D*, Hoechst 33258 staining.

agarose gel. The apoptosis was dependent on the DMJ treatment and concentration, and the incidence of apoptosis was increased in response to the level of MBQ-binding (Oki *et al.*, 1999a). These observations, together with an assumption that the mannosidase inhibitor DMJ does not affect the structures of cellular components except for the *N*-linked oligosaccharide, led to the conclusion that the cell surface glycoproteins are involved in apoptosis induced by MBQ.

MBQ distinguishes terminally exposed mannose residues from ones positioned internally. In the biosynthesis of *N*-linked (asparagine-linked) oligosaccharide, the core oligosaccharide $Glc_3Man_9GlcNAc_2$ is assembled on the lipid carrier dolichol pyrophosphate. After its en bloc transfer to nascent polypeptide chains in the endoplasmic reticulum, the carbohydrate part is further processed. Three glucose residues are removed by glucosidases I and II, giving rise to the high mannose type $Man_9GlcNAc_2$ core. Castanospermine inhibits this process by interfering with the glucosidases, which results in the accumulation of $Glc_3Man_9GlcNAc_2$. In mammals, further removal of mannoses and selective addition of a limited number of different monosaccharides (GlcNAc, Gal, Fuc, sialic acid) yield high-mannose hybrid or complex type structures. Swainsonine, a potent mannosidase II inhibitor, induces the accumulation of a hybrid type rather than a complex type glycan. After the preincubation of U937 cells in the presence of castanospermine or swainsonin, the cells did not show sensitivity to MBQ, and the binding level of FITC-labeled MBQ was as low as that of the untreated cells, suggesting that MBQ preferably binds to terminal mannose residues on glycoproteins (Oki *et al.*, 1999a).

B. Apoptosis Signaling Mediators: Reactive Oxygen Species and Calcium Ion

Reactive oxygen species (ROS) and calcium have been implicated as signaling molecules in apoptosis. A wide variety of cellular stresses such as TNF-α and cytotoxic drugs induce an increase in mitochondrial membrane permeability and the release of pro-apoptotic factors such as cytochrome *c* from the mitochondria. ROS, mainly produced by the mitochondria as a result of a dysfunction in the respiratory chain, mediate a death signal in apoptosis (Fleury *et al.*, 2002). Some antioxidants such as thioredoxine act as intracellular ROS scavengers and are capable of inhibiting the ROS-mediated apoptosis signaling. On the other hand, several previous studies have implicated an increase in cytosolic Ca^{2+} and a depletion of endoplasmic reticular Ca^{2+} in the initiation of apoptosis (McConkey and Orrenius, 1997). The

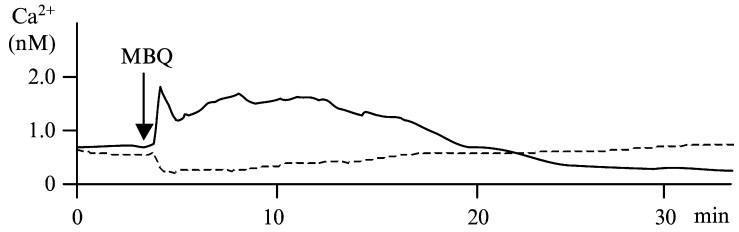


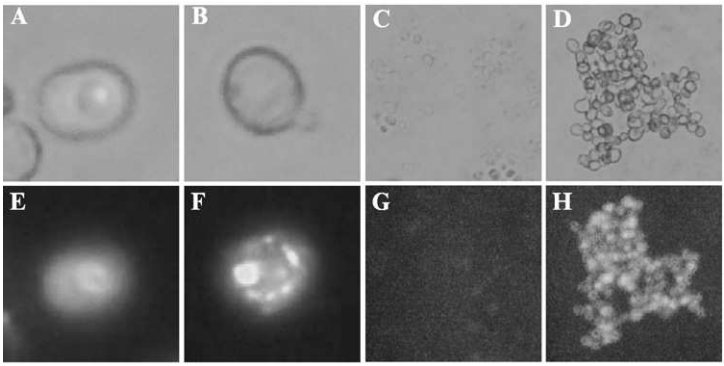
FIG. 3. Cytosolic Ca^{2+} level change in U937 cells after MBQ addition. Cytosolic Ca^{2+} concentration was measured by Fura2 fluorescence. 50 μg/ml MBQ was added to the cell culture at the time indicated by an arrow. Solid line: cells preincubated with DMJ for 48 hr, dashed line: control cells.

endoplasmic reticular Ca^{2+} depletion is caused by many apoptotic stimuli and occurs prior to release of cytochrome *c* from mitochondria (Szalai *et al.*, 1999). Similar to these findings, the intracellular Ca^{2+} level in the DMJ-preincubated U937 cells was elevated within a second in response to the exposure to MBQ, dropped to the normal level, and a subsequent gradual increase and decrease followed (Fig. 3, Oki *et al.*, 1999b). More importantly, MBQ induced the ROS accumulation in the cells ultimately resulting in the apoptosis. An antioxidant *N*-acetylcysteine (NAC) rescued the cells from apoptosis, indicating that ROS act as signaling molecules in MBQ-induced apoptosis.

III. Apoptosis-Like Yeast Cell Death Induced by MBQ

A. Apoptotic Morphology

MBQ shows fungicidal activity in a calcium-dependent manner by interacting with cell surface components that causes a perturbation of membrane function. Actually, the increase in membrane permeability by binding of MBQ was implicated by a rapid K^+ leakage from *C. albicans* cells (Sawada *et al.*, 1990). In addition, MBQ-treated *C. albicans* cells displayed pit-like invaginations in the plasma membrane, detachment of the cell membrane from the cell wall, fragmentation of nuclear and mitochondrial membranes, and multibudded morphology (Numata *et al.*, 1993). Our recent investigation revealed that the *Saccharomyces cerevisiae* cells show a phenotype with typical apoptosis markers that are reported in mammalian cells (Hiramoto *et al.*, 2003a). DAPI-staining of MBQ-treated *S. cerevisiae* cells revealed randomly distributed nuclear fragments, and the TUNEL stained the cells bright yellow-orange, indicating a strong DNA fragmentation



A/E and C/G: control. B/F and D/H: treated with 20 μg/ml MBQ for 2 h.
A-D: Nomarski optics. E and F: DAPI staining. G and H: TUNEL staining.

FIG. 4. DNA fragmentation in *S. cerevisiae* induced by MBQ. *A/E* and *C/G*, Control. *B/F* and *D/H*, Treated with 20 μg/ml MBQ for 2 hr. *A–D*, Nomarski optics. *E* and *F*, DAPI staining. *G* and *H*, TUNEL staining.

(Fig. 4, see color insert). On the other hand, most of the MBQ-treated cells were not stained with propidium iodide, implying the manner of this cell death was not necrosis.

B. APOPTOSIS OF YEAST

Apoptosis is a highly regulated process of programmed cell death and plays a central role in development and homeostasis of metazoan organisms. Apoptosis had been assumed confined to multicellular organisms because a suicide mechanism seemed useless for a unicellular organism that is unlikely to need socially advantageous regulation of cell survival. However, it is now believed that the basic machinery of apoptosis is present and functional in some unicellular eukaryotes. For example, the parasite *Trypanosoma cruzi*, the slime mold *Dictiostelium discoideum*, the ciliate *Tetrahymena thermophila*, the dinoflagellate *Peridinium gatunense*, and the yeast *S. cerevisiae* show the apoptotic phenotypes described in multicellular organisms—such as cytoplasmic blebbing and vacuolization, DNA fragmentation, and chromatin condensation—in response to environmental stress or extracellular signals (Ameisen, 1996; Madeo *et al.*, 2002). Interestingly, although yeast do not have apparent homologues of major apoptotic regulators, the heterologous expression of metazoan-derived apoptotic inducers including Bax, caspases or p53 results in an apoptotic death of *S. cerevisiae*, and anti-apoptotic Bcl-2 family proteins such as Bcl-2, Ced-9, and Bcl-x_L

prevent the Bax-induced death in yeast (Chen *et al.*, 2003; Madeo *et al.*, 2002). Yeast apoptosis can be induced by oxidative stress such as hydrogen peroxide or glutathione depletion (Madeo *et al.*, 1999) or by a point mutation in cell cycle gene *CDC48* (Madeo *et al.*, 1997).

C. ROS Signaling to Apoptosis

ROS are well established as inducers and mediators of apoptosis in mammals. In yeast, ROS generation is induced by radical inducers including hydrogen peroxide and menadione (Stephen *et al.*, 1995) or an antifungal agent such as miconazole (Kobayashi *et al.*, 2002), and ROS inactivating genes, including catalase, superoxide dismutase, or cytochrome *c* peroxidase, are induced by oxidative stress (Moradas-Ferreira *et al.*, 1996), osmotic stress (Garay-Arroyo *et al.*, 2003; Rep *et al.*, 2001), or heat shock (Davidson *et al.*, 1996), implying the induction of ROS generation by such stresses. Surprisingly, the exposure to MBQ induced the accumulation of ROS in *S. cerevisiae* cells. Moreover, the ROS accumulation and the cell death was inhibited by NAC, similar to the case of mammalian cell (Fig. 5, see color insert, Hiramoto *et al.*, 2003a). Several authors reported the protective effect of antioxidants or antioxidative proteins on ROS-mediated yeast cell death: the Cu,Zn-superoxide dismutase is required for resistance to osmotic shock (Garay-Arroyo *et al.*, 2003), Bcl-2 family proteins inhibit oxidative stress-induced cell death (Chen *et al.*, 2003), and the antifungal effect of miconazole is antagonized by an antioxidant (Kobayashi *et al.*, 2002). Consistent with the result obtained with U937 cells, it is thus concluded that the ROS are apoptosis signaling mediators in yeast death induced by MBQ.

IV. A Genetic Approach to the Action Mechanism of MBQ

The existence of potential target molecules of MBQ in the plasma membrane is implicated by several observations: the MBQ binding to cell surface is essential for the antifungal activity, MBQ induces a leakage of small molecules and invagination formation in plasma membrane surfaces, and MBQ shows its effectiveness to both *C. albicans* cells and protoplasts. To obtain the proteins involved in the fungicidal action of MBQ, mutant strains of *S. cerevisiae* that are resistant to MBQ were isolated on an agar plate containing MBQ after NTG treatment, and the genes that complement the resistance to MBQ were searched for by using the *S. cerevisiae* genomic library. So far we identified two genes, *YPD1* and *SSK1*, each of which encodes a protein

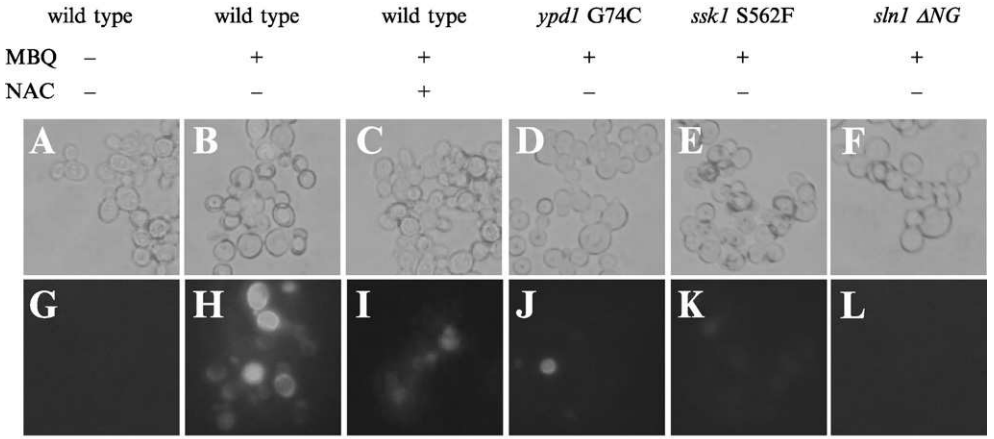


FIG. 5. ROS accumulation in *S. cerevisiae* induced by MBQ. *A/G*, Control; *B/H*, *D/J*, *E/K*, and *F/L*, treated with 20 μg/ml MBQ for 2 hr. *C/I*, Pretreated with 5 mM *N*-acetylcysteine before MBQ-treatment. *A–F*, Nomarski optics. *G–L*, 2,7-dihydrodichlorofluorescein staining.

involved in osmolarity regulation in *S. cerevisiae*, that are responsible for the resistance to MBQ (Hiramoto *et al.*, 2003b).

A. Osmoregulation in Yeast

In *S. cerevisiae*, osmolarity is regulated through multistep phosphoryl transfer reactions in which three transmembrane proteins—Sln1, Sho1, and Msb2—function as an osmosensor (Hohmann, 2002; Maeda *et al.*, 1994; O'Rourke and Herskowitz, 2002; O'Rourke *et al.*, 2002; Posas *et al.*, 1996). One branch of the pathway (Sln1-Ypd1-Ssk1, Fig. 6) is important in the response to hyperosmotic stress. Under hyperosmosis, the autophosphorylation activity of Sln1 is diminished and Ssk1 becomes a dephosphorylated active form. Then Ssk1 interacts with and activates the downstream HOG1 pathway, resulting in the translocation of Hog1 into the nucleus and the transcriptional induction of a large number of genes, some of which are responsible for the production of glycerol, an important compatible osmolyte in yeast. Under normal osmotic conditions, Sln1 becomes a phosphorylated form by autophosphorylation, and Ypd1, a histidine-containing phosphotransfer,

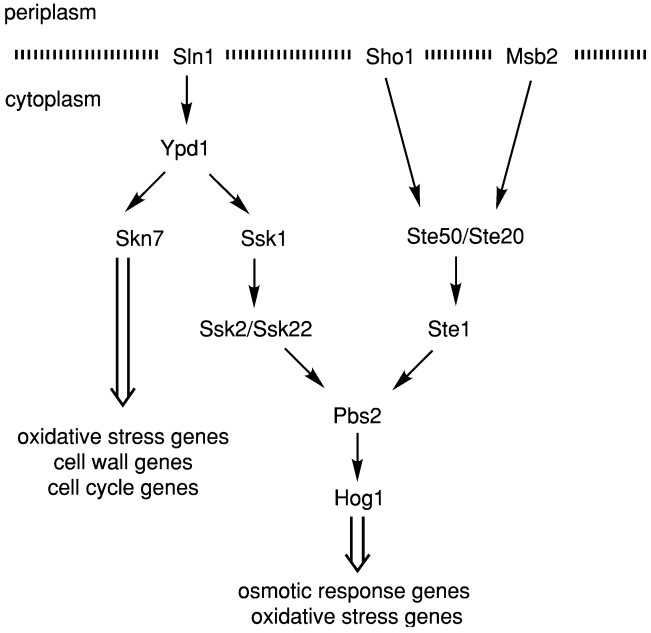


Fig. 6. Osmotic and oxidative stress response pathways in *S. cerevisiae*.

mediates the phosphoryl group transfer from Sln1 to a response regulator protein Ssk1 (Posas *et al.*, 1996). The phosphorylated Ssk1 is unable to activate the HOG1 pathway. The second branch of the pathway (Sln1-Ypd1-Skn7, Fig. 6) is activated in response to hypoosmotic stress (Tao *et al.*, 1999) and is implicated in the regulation of cell wall biosynthesis (Brown *et al.*, 1994) and the cell cycle (Morgan *et al.*, 1995). Under hypoosmosis, Sln1 becomes phosphorylated, which promotes the accumulation of the phosphorylated state of the response regulators, Ssk1 and Skn7 (Li *et al.*, 1998). The phosphorylated Skn7 is a transcription factor that activates the genes implicated in the response to oxidative damage (Krems *et al.*, 1996) and cell wall damage (Li *et al.*, 2002).

B. Osmoresponse Genes *YPD1* and *SSK1* Mutants are Resistant to MBQ

A substitution of the 74th amino acid residue from glycine to cysteine in Ypd1 renders the mutant resistant to MBQ (MIC: wild-type 1∼3 μg/ml, *ypd1* G74C $>$ 100 μg/ml) (Hiramoto *et al.*, 2003b). Ypd1 is a phosphotransfer protein that interacts with Sln1 to accept, and with Ssk1 and Skn7 to deliver, a phosphoryl group through the histidine residue at position 64 that is located within a receiver domain (Xu and West, 1999). In addition to the catalytic function, the receiver domain of Ypd1 functions to stabilize the phosphorylated state of Ssk1 through a protein-protein interaction (Janiak-Spens *et al.*, 2000). The glycine74, relatively positioned at +10 to the active-site histidine, is highly conserved among the proteins, including Ypd1, that possess histidine-containing phosphotransfer activity, suggesting its possible role in maintaining the activity. The importance of the glycine residue in Ypd1 protein folding is implicated by an observation that a heterologous expression of *ypd1*G74A mutant in *Escherichia coli* resulted in the production of an insoluble protein, whereas mutant proteins such as *ypd1* H64Q or *ypd1* Q86A were obtained in a soluble form (Janiak-Spens and West, 2000).

Ssk1 functions in the downstream regulation of Ypd1 responsible for the activation of the HOG1 pathway through an interaction with the downstream protein Ssk2. The C-terminal portion (amino acids 475–670) of Ssk1 functions as a receiver domain that is essential for the interaction with Ssk2 (Posas and Saito, 1998). A substitution of Ssk1 at 562nd amino acid residue from serine to phenylalanine also confers the mutant highly resistant to MBQ (MIC: $>$ 100 μg/ml) (Nomura, unpublished result). The mutation site is positioned close to the phosphorylation active site at aspartic acid554, which likely affects

the native function of Ssk1 and also Ypd1 (Posas *et al.*, 1996). In fact, both of the *ypd1* G74C and *ssk1* S561F mutants are hypersensitive to high osmolarity (e.g. 1.4 M NaCl), suggesting the abolishment of the HOG1 pathway in these mutants.

C. The HOG1 Pathway is not Involved in the Fungicidal Action of MBQ

The resistance to MBQ shown by *ypd1* G74C and *ssk1* S561F mutants appears to indicate the involvement of the HOG1 pathway in the action mechanism of MBQ. Activation of the HOG1 pathway leads to the transcriptional activation of not only the glycerol biosynthesis genes but also the oxidative damage protecting genes including hydroperoxide peroxidase and glutathione reductase (Rep *et al.*, 2001), which likely contributes to the reduction in ROS level and thus the protection from the ROS-mediated cell death induced by MBQ. Although the HOG1 pathway in the mutants *sln1* H576Q, *sln1* D1144N, and *ypd1* H64Q, in which the phosphorylation sites are inactivated, could be constitutively active because Ssk1 is maintained in an unphosphorylated active form, these mutants are highly sensitive to MBQ as well as the wild-type strain (Nomura *et al.*, 2003), implying that the HOG1 pathway is not related to the resistance to MBQ. On the contrary, the involvement of the HOG1 pathway in MBQ-resistance is implicated in a mutant of Ssk1 that does not have the conserved phosphorylation site at Asp554, *ssk1* D554N, which shows a moderate resistance to MBQ (MIC: 25$\sim$100 μg/ml) (Nomura, unpublished result). The *ssk1* D554N protein activates Ssk2 as well as the wild-type Ssk1 (Posas and Saito, 1998), suggesting that the HOG1 pathway is constitutively active in the *ssk1* D554N mutant, but the null mutants *hog1Δ* and *pbs2Δ* are sensitive to MBQ as well as the wild-type strain (Nomura *et al.*, 2003). These observations, together with the hypersensitivity of the MBQ-resistant mutants to high osmolarity, suggest that the HOG1 pathway is neither involved in resistance nor sensitivity to MBQ.

D. Osmosensor SLN1, a Presumptive Target of MBQ

It was first believed that MBQ causes an irreversible dysfunction of membrane permeability similar to amphotericin B that interacts with ergosterols in plasma membrane and abolishes membrane integrity. However, the action mechanism of MBQ is unlikely based on such an irreversible physical process because the wild-type *S. cerevisiae* grown in the medium supplemented with a lethal amount of MBQ can be

rescued by the antioxidant NAC. Further, the resistance to MBQ is achieved by a point mutation of a single gene *ypd1* or *ssk1*, indicating either the promotion or inhibition of a cellular process is sufficient to prevent the MBQ-induced cell death. The high resistance to MBQ in *ypd1* G74C or *ssk1* S561F mutants suggested the possible involvement of Sln1, an osmosensor that regulates the phosphorylation state of Ypd1 and Ssk1 in the upstream of the cascade. The membrane-spanning protein Sln1 has seven putative *N*-glycosylation sites in its extracellular domain. In *S. cerevisiae*, the *N*-glycosylation sites are modified with core oligosaccharides like $Man_9GlcNAc_2$, which are further modified by addition of an outer chain composed of an α-1,6-linked polymannose backbone and highly branched α-1,2- and α-1,3-side chains. Mutation of the seven putative *N*-glycosylation sites to which a polymannose *N*-linked glycan is not attached, confers the mutant *sln1 ΔNG* resistant to MBQ (MIC: 50∼100 μg/ml, Nomura *et al.*, 2003), indicating that the binding of MBQ to the *N*-linked glycans on Sln1 protein is responsible for the yeast death. Hence, it is likely that the Sln1 signal transduction output determines the fate of the yeast cell in the MBQ-induced apoptosis. Of the two signal transduction pathways regulated by Sln1, the SKN7 pathway appears to be the target of MBQ because several lines of evidence suggest the irrelevance of the HOG1 pathway in the fungicidal action of MBQ. Furthermore, the activation of the SKN7 pathway is unlikely a cause for the yeast death because the *skn7* null mutants are highly sensitive to MBQ (Nomura, unpublished result). Therefore, the most probable explanation for the mechanism of action of MBQ is that MBQ induces ROS accumulation through perturbing the membrane function and simultaneously inhibiting the activation of the SKN7 pathway essential for protecting the cell from ROS damage. In the extracellular domain, Sln1 has 62 serine and threonine residues that can be modified with *O*-linked oligomannose carbohydrate chains. The binding of MBQ to these *O*-linked glycans also appears to affect the Sln1 signal transduction, which likely explains a slightly lower resistance of *sln1 ΔNG* to MBQ.

E. A Possible Role of the SKN7 Pathway in MBQ Resistance

ROS are a normal by-product of respiring cells and are also produced by a wide range of different environmental chemicals. Hence, cells have developed cellular antioxidants and enzymes capable of rapidly detoxifying active oxygen. Active oxygen species, including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•), are noted for their high reactivity and resultant damage to DNA,

proteins, and cellular structures. In *S. cerevisiae*, several genes have been identified and shown to be involved in resistance against oxidative stress (Krems *et al.*, 1996; Moradas-Ferreira *et al.*, 1996). Although little is known regarding the signal transduction pathways that detect and respond to oxidative stress, the transcription factors Skn7 (Krems *et al.*, 1996; Morgan *et al.*, 1997) and Yap1 (Costa *et al.*, 2002; Stephen *et al.*, 1995) have been implicated in a cellular pathway that controls the oxidative stress response.

Skn7 is regulated in part by the Sln1 osmosensor through a phosphotransfer via Ypd1 to its conserved aspartic acid residue. Skn7 is capable of specific DNA binding and is responsible for the expression of several important oxidative stress genes (Lee *et al.*, 1999). Disruption of the SKN7 gene results in yeast cells becoming sensitive to oxidizing agents such as hydrogen peroxide, cadmium, and menadione, indicating that the Skn7 protein is required for the cellular response to a variety of free radicals (Morgan *et al.*, 1997). Skn7 controls the induction of at least nine antioxidant proteins in response to hydrogen peroxide (Lee *et al.*, 1999). Independent of Skn7, Yap1 regulates the induction of a variety of antioxidant defense enzymes but requires the presence of Skn7 for the expression of some oxidative stress genes. For example, Skn7 and Yap1 cooperate on the TRX2 promoter to induce transcription of *TRX2* and *TRR1*, encoding thioredoxin and thioredoxin reductase, respectively (Morgan *et al.*, 1997).

The ROS accumulation induced by MBQ is lethal to *S. cerevisiae*, and NAC rescues the yeast cell through scavenging the free radicals. Quite impressively, the MBQ-induced ROS accumulation is suppressed in the MBQ-resistant mutants *ypd1* G74C, *ssk1* S561F, and *sln1 ΔNG* (Fig. 5), implying that these mutants activate the defense response to oxidative damage caused by MBQ (Nomura *et al.*, 2003). These observations led to a model for the resistance mechanism in which Skn7 activates the gene expression of antioxidative enzymes through the activation of its phosphorylation by *ypd1* or *ssk1* mutant protein or as a result of the lower sensitivity to MBQ of *sln1 ΔNG*.

MBQ-treated *C. albicans* cells display a multibudding morphology (Numata *et al.*, 1993). A similar morphology is also observed in the *och1* null mutants of *S. cerevisiae* (Lee and Elion, 1999; Mondesért *et al.*, 1997). *OCH1* encodes an α-1,6-mannosyltransferase involved in *N*-linked glycoprotein maturation (Lehle *et al.*, 1995; Nakanishi-Shindo *et al.*, 1993), and its gene expression is regulated by the phosphorylated Skn7 (Li *et al.*, 2002). The similarity in the morphology of the MBQ-treated yeast cells and the *och1* null mutants might suggest a link of Sln1, a presumptive target of MBQ to Skn7.

V. Conclusion and Future Prospects

In conclusion, our current model for the action mechanism of MBQ is proposed based on the hypothesis that MBQ has dual functions in executing its fungicidal action (Fig. 7). First, the action mechanism of MBQ is essentially related to its binding to mannose-containing carbohydrate chains on membrane proteins. Several lines of evidence suggest that the primary target of MBQ is a membrane protein responsible for the transportation of small molecules such as a transporter or ion channel. The binding of MBQ causes a leakage of small molecules and ions which results in a perturbation of the cytosolic component homeostasis, which eventually induces a ROS leakage from mitochondria leading to the apoptosis-like death. Second, in parallel to the induction of ROS accumulation, MBQ likely inhibits the Sln1 function of phosphorylating Skn7 by binding to the *N*-linked glycans on Sln1, which perhaps results in a conformational change of the protein. The altered conformation enforces the accumulation of unphosphorylated Skn7, which is unable to respond to the oxidative stress. Conversely, in the resistant mutants *ypd1* G74C and *ssk1* S562F, the phosphorylated Skn7 level is increased as a result of the alteration of the phosphotransfer

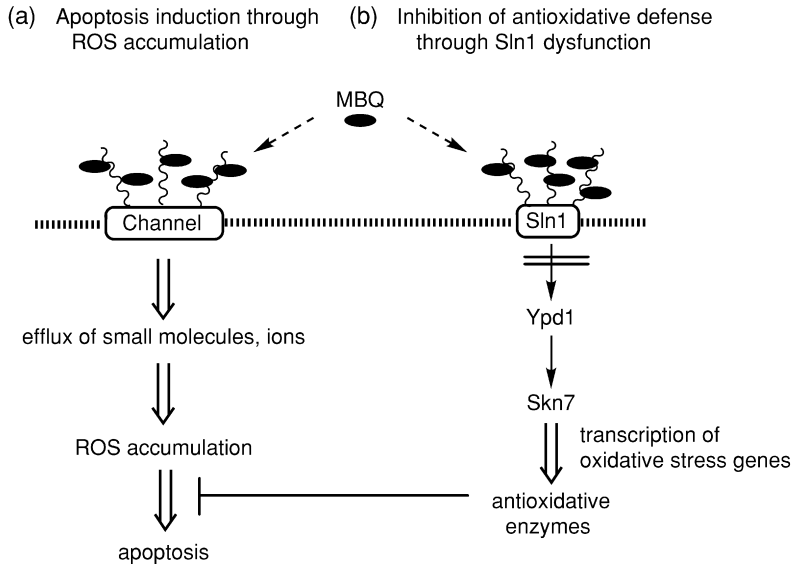


FIG. 7. A model for action mechanism of MBQ.

activity and/or protein-protein interaction in the mutant proteins, which enhances the expression of antioxidative proteins to protect the cell from ROS damage. The *sln1 ΔNG* mutant that lacks the polymannose *N*-linked glycans, the important binding site of MBQ, is less affected by MBQ and thus retains the activity for the Skn7 phosphorylation, which renders itself resistant to MBQ. Further investigations are in progress that will prove this hypothesis.

MBQ has an ideal profile for an antifungal agent, with high selectivity, fungicidal activity, low toxicity, and broad spectrum. Although the development of BMS-181184 and benanomicin has been withdrawn for unknown reasons, the MBQ derivative is believed to be one of the most promising candidates for the development in the near future.

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Protozoan Grazing of Freshwater Biofilms

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I. Introduction

It is currently estimated that 99% of microbial activity in freshwater exists within surface-associated communities (Bryers, 1982). These ''biofilms'' are thus perceived as being hot spots for biotic interactions, genetic exchange, and the biogeochemical cycling of elements. The most recent definition of a biofilm is ''a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription'' (Donlan and Costerton, 2002). Even though biofilms, or

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Aufwuchs, comprise a dynamic community of microbial cells, the majority of research has focused on the bacterial component. Some research has been directed at the attached algal community, which although termed ''periphyton,'' is in fact a component of biofilms. Less than 80 papers address the protozoan component of biofilms, and 38% of these have simply mentioned the presence of protozoa in the samples or have referred to the role of protozoa in harboring pathogens such as *Legionella*. This paucity of information is surprising, because protozoa are known to be the major consumers of bacteria in the plankton (Sherr *et al.*, 1983). The lack of research could be due to (i) the long-held belief that bacteria are afforded refuge from grazing by being embedded within the exopolymer matrix (Costerton *et al.*, 1981), which has since been disproved (Heaton *et al.*, 2001) and (ii) the lack of techniques for determining protozoan grazing rates on attached prey, which contrasts with the wealth of methods available for determining rates on suspended prey (e.g., Landry *et al.*, 1991; McManus and Fuhrman, 1986; Parry *et al.*, 2001; Sherr *et al.*, 1987).

II. Protozoan Form and Function

A. What Are Protozoa?

Free-living protozoa are single-celled eukaryotic organisms, normally of the size range 2–200 μm. Although some species are autotrophic, this review is concerned with those that are heterotrophic. Like most microorganisms, they have very large population sizes because of their short generation times and are thus the most abundant group of phagotrophic organisms in the biosphere (Finlay, 2001). They exhibit a variety of mechanisms to capture their prey, and this has led to a considerable diversification of protozoan morphologies. However, for simplicity sake they can be divided into amoebae, flagellates, and ciliates.

1. *Amoebae*

The naked amoebae move by projecting pseudopodia and crawling and are thus more associated with surfaces than the plankton. They are raptorial feeders and envelop their prey within pseudopodia before enclosing them in a food vacuole. Some amoebae are housed in a shell (testate amoebae) and feed by diffusion feeding whereby extrashellular cytoplasm is protruded through holes in the shell, prey are caught on the sticky cytoplasm, and then they are brought to the main body of the cell where they are digested. Amoebae cannot feed effectively on suspended prey (Rogerson and Laybourn-Parry, 1992) but are known to feed on attached bacteria at rates of between 0.2–1465 bacteria

amoeba^{-1} h^{-1} (Butler and Rogerson, 1997; Heaton *et al.*, 2001; Mayes *et al.*, 1997; Rogerson *et al.*, 1996).

2. *Flagellates*

Flagellates posses one or more flagella that are used for swimming and creating feeding currents. Water, containing prey, is drawn toward the base of the flagellum, where the prey are ingested via pseudopodia or are drawn into an oral groove. Some flagellates are filter feeders and have a collar of tentacles at the base of the flagellum that allows only the smallest of prey particles to pass through. Flagellates exhibit prey size-selective grazing because of their small size (2–20 μm) and the fact that only one prey is enclosed within a single food vacuole (Chrzanowski and Šimek, 1990; Matz *et al.*, 2002). They are therefore considered the dominant predators of bacteria in aquatic systems (Sanders *et al.*, 1989; Sherr *et al.*, 1983), with ingestion rates of 2–300 bacteria flagellate^{-1} h^{-1}, with the majority of values being $<$70 (Chrzanowski and Šimek, 1990; Davies and Seiburth, 1984; Eccleston-Parry and Leadbeater, 1994). The smaller bacteria ($<$1.6 μm) are generally ''grazing vulnerable,'' but larger cells ($>$2.4 μm) are considered ''grazing resistant'' (Pernthaler *et al.*, 1996). Flagellates may be free-swimming in the plankton (e.g., *Monas*) or attached to a surface either by a trailing flagellum (e.g., *Bodo*) or a stalk (e.g., *Pteridomonas*) and the choanoflagellates. By being attached, the flagellates can use their flagellum more efficiently to create larger feeding currents, thus increasing their rates of ingestion of suspended prey (Fenchel, 1982).

3. *Ciliates*

Ciliates comprise another protozoan group that feeds effectively on bacteria (Sherr and Sherr, 1987), but their larger cell sizes allow them to exploit more prey types such as algae, flagellates, and other ciliates (Bernard and Rassoulzadegan, 1991; Dolan and Coats, 1991; Epstein *et al.*, 1992; Stoecker and Evans, 1985). Ciliates are the most diverse group with regard to their feeding behaviors, although all but one group, the suctorian ciliates, are filter feeders. They possess cilia for swimming and shorter hairs, called *membranelles*, for feeding. Water is drawn to the oral area (cytostome), where many prey cells are deposited within a single food vacuole. Ciliate ingestion rates are thus notoriously high, reaching values of 1254 bacteria ciliate^{-1} h^{-1} (Iriberri *et al.*, 1995). Ciliates can be found freely swimming in suspension (e.g., *Tetrahymena*), crawling on surfaces (e.g., *Euplotes*), or can be physically attached to surfaces, usually by means of a stalk (e.g., *Vorticella*).

TABLE I

CLASSIFICATION OF PROTOZOA BASED ON THEIR LEVELS OF INTERACTION WITH SURFACES

Protozoan group	Characteristics
Transient	Predominantly free-swimming, feeding on suspended prey
Sessile	Attached to surface, feeding on suspended prey
Browser	Free-swimming, feeding on suspended prey and browsing over surfaces, feeding on attached prey
Amoebae	Browsing over surfaces, feeding on attached prey only

Once again, those that are attached to a surface produce large feeding currents and are effective grazers of suspended prey cells.

4. *Diversity of Protozoan in Biofilms*

Members of all three protozoan groups have been found in freshwater biofilms (e.g., Hunt and Parry, 1998; Jax, 1996; Madoni, 1994; Pérez-Uz *et al.*, 1998; Pratt *et al.*, 1986; Strüder-Kype, 1999), but their diversity warrants a review of their own. Protozoan composition in biofilms is similar to those of planktonic aggregates, so the reader is directed to a review by Zimmermann-Timm, (2002) and the references within.

Although many different protozoan species are found in association with biofilms, their level of association and ultimate grazing impact on the biofilm-prey will differ. In light of this, biofilm-associated protozoa are separated into four groups for the purpose of this review (Table I). These groups include those that are predominantly planktonic but can swim close to the biofilm (''transient protozoa''), those that use it as a surface for attachment but feed on suspended prey (''sessile protozoa''), and those that feed directly on biofilm-associated prey (''browsers'' and ''amoebae'').

B. WHAT IS THE ECOLOGICAL ROLE OF PROTOZOA

1. *The Function of Protozoa in the Plankton*

The concept of the ''microbial loop'' (Azam *et al.*, 1983) is now widely accepted as being an important component of the classical food chain. Briefly, bacterial production is fuelled by dissolved organic carbon (DOC), which is considered to be derived mainly from phytoplankton photosynthate (Bird and Kalff, 1984; Vadstein *et al.*, 1989). Both the bacteria and algae are grazed on by the protozoa, and with

their short generation times they quickly affect the growth dynamics of their prey. Protozoa can remove between 30–100% of bacterial production per day (Sherr *et al.*, 1983) and maintain their prey in a ''physiological state of youth'' (Johannes, 1965). The protozoa are then grazed by meso- and macro-invertebrates, and carbon is transferred throughout the food web. In addition to carbon cycling, protozoa play a crucial role in the cycling of nitrogen (N) and phosphorus (P). They remineralize the organic N and P within their prey to soluble, inorganic N and P, which is excreted mainly as ammonium and orthophosphate, respectively (Alldredge and Cohen, 1987; Eccleston-Parry and Leadbeater, 1995; Goldman *et al.*, 1985). These nutrients are then utilized by their prey.

2. *The Function of Protozoa in the Biofilms*

There is little information on the role of protozoa in biofilms. There is even a dearth of information regarding bacterial-algal interactions on surfaces, but it does appear that they mirror those of the plankton. For example, bacteria and algae have been shown to be coupled in biofilms, indicating bacterial dependence for algal exudates (Haack and McFeters, 1982; Hunt *et al.*, 1999; Stock and Ward, 1989). The uncoupling of bacteria from algae has also been detected in biofilms, with the bacteria utilizing an alternative carbon source (e.g., humic substances) (Hessen, 1985; Hunt *et al.*, 1999) as they also do in the plankton of brown-water lakes (Jones and Salonen, 1985; Tranvik, 1989). It is unclear whether bacteria utilize the biofilm matrix as a source of nutrients. The long-held belief is that it is recalcitrant (Christensen and Characklis, 1990), but recent evidence suggests that it may indeed be biodegradable (Battin *et al.*, 1999; Zhang and Bishop, 2003).

The similarities between bacterial-algal interactions in biofilms and the plankton imply that protozoan interactions in biofilms might also be similar to that occurring in the plankton, for which there is a wealth of information. This chapter reviews the corresponding information on protozoa in biofilms, such as their grazing rates, evidence of nutrient remineralization, and their susceptibility to mesoinvertebrate grazing. Differences in protozoan behavior in the biofilm, compared with the plankton, are also expected because of the different protozoan community structure in biofilms (i.e., the prevalence of sessile forms and amoebae). In addition, the close proximity of microbial components could lead to increased levels of cell–cell signalling and increased interaction with pathogenic bacteria, which are harbored within freshwater biofilms.

III. Protozoan Grazing of Biofilms

It has been over 10 years since Pederson (1990) stated that the effects of protozoan grazing on freshwater biofilms was probably the most important factor controlling biofilm dynamics, which was often overlooked in studies. Yet data on their involvement in biofilm processes is still scarce. Much of the earlier work on protozoan grazing of attached bacteria came from marine aggregate studies (Albright *et al.*, 1987; Caron, 1987; Sibbald and Albright, 1988) and benthic systems (Kemp, 1988; Starink *et al.*, 1994). Parallel research in freshwater systems was more qualitative, such as assessing the level of sloughing by amoeboid movement and the subsequent colonization of bare patches by choanoflagellates (Jackson and Jones, 1991) and peritrich ciliates (Harmsworth and Sleigh, 1993). Evidence that freshwater protozoa actually ingested biofilm-associated bacteria was first reported by Wolfaardt *et al.* (1994), though indirectly.

A. Bacterial Phenotypes in Biofilms

Bacteria within biofilms exhibit a range of phenotypes, many of which do not exist in the plankton. Six phenotypes are hypothesised here (Table II), and each will differ in the extent to which they are perceived as suitable prey by the protozoa. Briefly, when suspended bacterial cells make contact with a substratum, they initially undergo ''reversible adhesion'' and are easily removed from the surface, resuming their planktonic state (''suspended, naked cells''). After this reversible adhesion, firm adhesion of the bacterial cells occurs, and they are

TABLE II

Classification of Bacterial Cell States Associated With Biofilms

Bacterial prey form	Characteristics
1. Suspended, naked	Freely suspended naked cells and those reversibly attached to a surface
2. Recently attached	Irreversibly attached to a surface and naked
3. Embedded	Attached cells surrounded by EPS or capsule
4. Deeply embedded	Attached cells within a microbial stack, surrounded by matrix
5. Suspended, coated	Capsulated, free-swimming cells or embedded cells sloughed into suspension
6. Hydrophilic daughters	Naked, suspended daughter cells

not removed by moderate shear force (''recently attached cells''). The cells then grow and exude exopolysaccharides (EPS), which accumulate around the cell and coat them (''embedded cells''). Further growth leads to cell division and the development of microcolonies, in which the cells are enveloped in the gel-like matrix, and some are rooted firmly within the bacterial stack (''deeply embedded cells''). These stacks are separated by water channels that allow the exchange of nutrients and waste products with the bulk fluid phase, but they may also allow the movement of protozoa to the deepest parts of a biofilm. When the embedded bacteria divide, they produce hydrophilic daughter cells that are propelled from the biofilm, only being able to attach to a surface after three generations when they become more hydrophobic (''hydrophilic daughter cells'') (Allison *et al.*, 1990). Finally, cells may detach or be sloughed off the surface, forcing matrix-coated cells into suspension (''suspended, coated cells''). The latter prey form may also comprise those planktonic bacteria that inherently posses a capsule.

B. Gross Grazing Rates on Surface-Associated Prey

Studies on the ingestion of attached bacteria by protozoa *per se* have been mainly carried out on benthic systems, as it is generally considered that benthic bacteria are predominantly attached to sediment particles. Experiments that have added fluorescently labelled bacteria (FLB) stained with 5-([4,6-dichlorotriazin-2-YL]amino)-fluorescein (DTAF) (Sherr *et al.*, 1987) to sections of intact sediment cores have yielded flagellate grazing rates of 0–104 FLB $cell^{-1}$ h^{-1} (Epstein and Shiaris, 1992; Hondeveld *et al.*, 1992). Higher values of 37–421 FLB $cell^{-1}$ h^{-1} have been recorded for ciliates; mainly *Euplotes* and tintinnids (Kemp, 1988). Even so, many of these added FLB might still have been present in the pore-water because of the short incubation times used (30–60 min), yielding estimates of protozoan grazing on both attached and suspended prey simultaneously. When the sediment itself is stained with DTAF, to stain only the attached bacteria before adding it to fresh sediment, a much lower ingestion rate of 61 FLB $protozoan^{-1}$ h^{-1} is recorded (Starink *et al.*, 1994).

C. Grazing Rates of Sessile and Transient Ciliates

1. *Grazing Rates of Sessile Ciliates on Suspended Prey*

Sessile ciliates, by their very nature, would be expected to feed avidly on suspended prey cells (Table I) and to date, most work on grazing of these protozoa has employed suspended, naked prey (Table II).

Carrias *et al.* (1996) added fluorescently-labelled microspheres (FLM) (McManus and Fuhrman, 1986) to river water and determined the grazing rates of different protozoan genera after a 15 minute incubation period. The only sessile ciliates which consumed the suspended particles were two species of *Vorticella*, having cell lengths of 48 and 24.5 μm, which consumed between 460–5910 and 30–1610 FLM cell^{-1} h^{-1}, respectively. Another peritrich ciliate, *Epistysis* (cell length ca. 30 μm), has been shown to ingest 1209 FLM cell^{-1} h^{-1} during 30 minutes incubation (Eisenmann *et al.*, 2001). The prey particles used in Eisenmann's study were an equal mixture of Green hydrophobic- and Red hydrophilic-FLMs, and the food vacuoles of *Epistysis* contained equal numbers of both particle type. This suggests that hydrophilic daughter cells (Table II) should be ingested at equivalent rates to the more hydrophobic suspended, naked cells.

2. *Grazing Rates of Transient Ciliates on Suspended Prey*

The study of Carrias *et al.* (1996) highlighted those ciliates that could possibly be ''transient'' members of the aggregate/biofilm (Table I). The incubation period was only 15 minutes, so there would be little time for FLM to attach to the clay particles *and* be grazed on by browsing species. They would more likely be ingested by protozoa that feed effectively on suspended prey. These presumptive transients included a free-swimming peritrich with an ingestion rate of 200 FLM cell^{-1} h^{-1}, members of the Oligotrichida, *Pelagohalteria viridis, Stombilidium* spp., and *Halteria* sp., which had ingestion rates of 110–1320, 6–130 and 12–440 FLM cell^{-1} h^{-1}, respectively, and members of the Scuticociliates, *Uronema* sp., and *Cyclidium* sp., with ingestion rates of 5.8–320 and 14–64 FLM cell^{-1} h^{-1}. Indeed, scuticociliates are known to preferentially feed on suspended prey (Albright *et al.*, 1987; Tso and Taghon, 1999) and they have been shown, along with numerous other transient ciliates, to consume suspended *E. coli* cells coated in alginate (ca. Prey form 6, Table II) (Heaton, unpublished data). As for the ingestion of hydrophilic daughter cells, Tso and Taghon (1999) found that *Cyclidium* selected for more hydrophobic prey over hydrophilic, thus the effect of prey cell hydrophobicity might be species-specific in ciliates, as no selection was evident with the sessile *Epistysis* (Eisenmann *et al.*, 2001).

3. *Grazing Rates of Sessile and Transient Ciliates on Sloughed Prey*

The swimming behavior of transient ciliates and the extensive feeding currents produced by both sessile and transient ciliates might be strong enough to detach recently attached bacterial cells and possibly

embedded cells (Table II), thus allowing the protozoa to feed on these suspended prey. Albright *et al.* (1987) demonstrated that although *Uronema* sp., a scuticociliate and a free-swimming peritrich, preferentially fed on suspended FLB, they could still feed on bacteria attached to calcium-alginate beads at rates of 1.4, 16.7, and 16.4 FLB $cell^{-1}$ h^{-1}, respectively (Table III). Zubkov and Sleigh (1999b) also showed that *Uronema marinum* could feed indirectly on live *Vibrio natriegens* deposited onto filters, but Tso and Taghon (1999) found that *Cyclidium* sp. could only detach and consume DTAF-stained *Vibrio* sp. in one out of five experiments. Even so, the ciliate had a grazing rate of 63 FLB $cell^{-1}$ h^{-1} (Table III). Higher ingestion rates of sloughed cells have been determined for *Tetrahymena* sp. feeding on *Pseudomonas putida* attached to glass beads (880 bacteria $cell^{-1}$ h^{-1}), although this was half the value of that recorded when the ciliate was feeding on suspended *Ps. putida* (1382 bacteria $cell^{-1}$ h^{-1}) (Eisenmann *et al.*, 1998). Leung *et al.* (2000) showed that even though *Tetrahymena thermophila* could graze on suspended GFP-expressing *Moraxella* at rates of 470 bacteria $cell^{-1}$ h^{-1}, they could not ingest any bacterial cells when they were encapsulated in alginate beads (Table III). Therefore, in their study the bacterial cells were essentially embedded into the alginate (Prey forms 3 and 4, Table II), whereas in the other studies they were attached to the outer surface of particles (Prey form 2, Table II), being possibly more susceptible to detachment by the ciliates.

D. Grazing Rates of Sessile and Transient Flagellates on Suspended Prey

The study of Carrias *et al.* (1996) showed that sessile flagellates ingested FLM rapidly during the 15-minute incubation period with ingestion rates of 1.7–33.6 and 6–33.6 FLM $cell^{-1}$ h^{-1} for Choanoflagellates and Bicoecids, respectively. The results also suggested that the flagellate *Monas* was an important transient genus, with grazing rates of 1.6–27 FLM $cell^{-1}$ h^{-1}. Indeed, Caron (1987) found that *Monas* sp. was unable to feed on *Pseudomonas halodurans* attached to chitin particles but was an avid grazer of the prey in suspension. He also showed that *Cryptobia* sp. could feed only on suspended prey, even though it is physiologically designed to browse on surfaces. *Pteridomonas danica* is a sessile flagellate that feeds effectively on suspended prey, with Gross Growth Efficiencies (GGE) between 50 and 60% (Zubkov and Sleigh, 2000). But this flagellate was also shown to feed on 55% of a population of *Vibrio natriegens* deposited onto filters. It appears that the creation of feeding currents sloughed the prey cells into suspension, where they were

TABLE III

PUBLISHED INGESTION RATES OF BIOFILM-ASSOCIATED PROTOZOA FEEDING ON ATTACHED BACTERIA

Protozoan	Prey	Prey Concentration	Ingestion Rate (prey cell^{-1} h^{-1})	Temp (°C)	Source
Mixed Protozoa	DTAF-sediment	5.33×10^9 cells cm^{-3}	61.3	18	Starink *et al.* (1994)
Mixed Flagellates	FLB (mixed)	$4.5–5.5 \times 10^8$ cells cm^{-3}	2.5–11.8	?	Honderveld *et al.* (1992)
Mixed Flagellates	FLB (coliforms)	ca. 1×10^8 cells cm^{-3}	1.2	22	Epstein and Shiaris (1992)
Mixed Ciliates	FLB (single sp.)	ca. $4–8 \times 10^8$ cells cm^{-3}	37–421	?	Kemp (1988)
Ciliates					
Chlamydodon sp.	FLB (coliforms)	ca. 1×10^8 cells cm^{-3}	162	22	Epstein and Shiaris (1992)
Cyclidium sp.	FLB (*Vibrio*)	ca. 10^8 cells cm^{-3}	63	20	Tso and Taghon (1999)
Euplotes sp.	FLB (mixed)	$3–6 \times 10^5$ cells cm^{-3}	12.4	25	Allbright *et al.* (1987)
Euplotes sp.	*Pseudomonas*	ca. 3×10^5 cells cm^{-2}	882	?	Lawrence and Snyder (1998)
Euplotes sp.	*Vibrio*	ca. 1×10^5 cells cm^{-2}	120	?	Lawrence and Snyder (1998)
Euplotes sp.	FLB (*Vibrio*)	ca. 10^8 cells cm^{-3}	3–176	20	Tso and Taghon (1999)
Pertrich ciliate (swimming)	FLB (mixed)	$3–6 \times 10^5$ cells cm^{-3}	16.4	25	Allbright *et al.* (1987)
Prorodon sp.	FLB (coliforms)	ca. 1×10^8 cells cm^{-3}	169	22	Epstein and Shiaris (1992)
Scuticociliate	FLB (mixed)	$3–6 \times 10^5$ cells cm^{-3}	16.7	25	Allbright *et al.* (1987)
Tetrahymena thermophila	GFP- *Moraxella*	$2–8 \times 10^8$ cfu cm^{-3}	0	22	Leung *et al.* (2000)
Tetrahymena sp.	*Pseudomonas*	8×10^5 cells cm^{-2}	648–880	25	Eisenmann *et al.* (1998)
Uronema sp.	FLB (mixed)	$3–6 \times 10^5$ cells cm^{-3}	1.4	25	Allbright *et al.* (1987)

Amoebae					
Clydonella rosenfieldi	FLB (*Planococcus*)	2.5×10^8 cells cm^{-3}	2.9	20	Butler and Rogerson (1997)
Dactylamoeba sp.	FLB (*Planococcus*)	2.5×10^8 cells cm^{-3}	76.5	20	Butler and Rogerson (1997)
Hartmannella cantabrigiensis	GFP-*E. coli (emb)*	7×10^6 cells cm^{-2}	243 (136)	20	Heaton *et al.* (2001)
Isolate 5	FLB-Gram -ve	$4–350 \times 10^6$ cells cm^{-3}	0.86–34.59	4	Mayes *et al.* (1997)
Paraflabellula reniformis	FLB (*Planococcus*)	2.5×10^8 cells cm^{-3}	6.9	20	Butler and Rogerson (1997)
Platyamoeba autralis	FLB-Gram -ve	$4–350 \times 10^6$ cells cm^{-3}	1.2–28.6	4	Mayes *et al.* (1997)
Platyamoeba placida	GFP-*E. coli (emb)*	7×10^6 cells cm^{-2}	15 (17)	20	Heaton *et al.* (2001)
Platyamoeba sp.	FLB (*Planococcus*)	2.5×10^8 cells cm^{-3}	0.2	20	Butler and Rogerson (1997)
Saccamoeba limax	GFP-*E. coli (emb)*	7×10^6 cells cm^{-2}	58 (0)	20	Heaton *et al.* (2001)
Stereomyxa ramose	FLB (*Planococcus*)	ca 1.4×10^7 cells cm^{-2}	ca. 111	20	Rogerson *et al.* (1996)
Synamoeba arenaria	FLB (*Planococcus*)	ca 1.4×10^7 cells cm^{-2}	ca. 208	20	Rogerson *et al.* (1996)
Trichosphaerium sieboldi	FLB (*Planococcus*)	ca 1.4×10^7 cells cm^{-2}	1465	20	Rogerson *et al.* (1996)
Unidentified filose	FLB (*Planococcus*)	ca 1.4×10^7 cells cm^{-2}	10	20	Rogerson *et al.* (1996)
Vahlkampfia avara	GFP-*E. coli (emb)*	7×10^6 cells cm^{-2}	440 (434)	20	Heaton *et al.* (2001)
Vahlkampfia damariscottae	FLB (*Planococcus*)	2.5×10^8 cells cm^{-3}	9.7	20	Butler and Rogerson (1997)
Vannella caledonica	FLB (*Planococcus*)	2.5×10^8 cells cm^{-3}	2.2	20	Butler and Rogerson (1997)
Vexillifera bacillipedes	GFP-*E. coli (emb)*	7×10^6 cells cm^{-2}	16 (2)	20	Heaton *et al.* (2001)

emb: E. coli cells embedded in 3% calcium-alginate.

consumed. Evidence for this was an obvious delay in the growth response of the flagellate compared to that when feeding on suspended prey from the onset (Zubkov and Sleigh, 1999a). *Paraphysomonas imperforata* has also been seen to slough *V. natriegens* off filters and then consume the cells (Zubkov and Sleigh, 1999b).

Many flagellate species, including *Paraphysomonas*, attach to surfaces temporarily by means of a stalk, and hence it is difficult to classify them as predominantly transient or sessile. However, they still preferentially feed on suspended prey (Sala and Güde, 1999; Sibbald and Albright, 1988). A number of studies have thus examined the ingestion of suspended prey by flagellates that have been attached to a glass. Boenigk and Arndt (2000a,b) showed ingestion rates of 14.1, 36.7, 63, and 24 bacteria cell^{-1} h^{-1} for attached *Cafeteria roenbergensis, Spumella* sp., *Ochromonas* sp., and *Monosiga* sp. feeding on suspended *Ps. putida*. The hydrophobicity of the prey cell has been shown not to affect ingestion rates of flagellates (Matz and Jürgens, 2001; Matz *et al.*, 2002), and Matz *et al.* (2002) also showed that *Spumella* sp. could consume capsulated bacteria if cell dimensions (including the capsule) were $\leq$3.3 μm $\times$ 1.08 μm.

E. Grazing Rates of Amoebae on Attached Prey

Amoebae can only graze effectively on attached prey (Rogerson and Laybourn-Parry, 1992), thus they have a choice of three prey forms (Forms 2–4, Table II). Very little data are available on the feeding of testate amoebae, although it is becoming clear that most of the larger species may not preferentially feed on bacteria (Epstein and Shiaris, 1992) but feed on larger ciliates, rotifers, and smaller testate amoebae (Gilbert *et al.*, 2003). However, Rogerson *et al.* (1996), who were the first to determine ingestion rates for amoebae grazing on a surface-associated bacterium, found that the testate amoeba *Trichosphaerium sieboldi* (60 μm in size) ingested 1465 *Planococcus citreus* cells amoeba^{-1} h^{-1}. Ingestion rates for the naked amoebae were lower (Table III). Butler and Rogerson (1997) improved the method for the determination of amoebic ingestion rates and dried DTAF-stained *Planococcus citreus* onto agar, thereafter adding the predator and viewing cells up to 40 min. Table III shows the results of experiments performed at 20 °C, where ingestion rates ranged from 0.2 to 76.5 bacteria cell^{-1} h^{-1} for *Platyamoeba* sp. and *Dactylamoeba* sp., respectively. Mayes *et al.* (1997) used the same method and prey, but at temperatures $\leq$4 °C, to assess ingestion rates of two Antarctic amoebae, *Platyamoeba australis* and an isolate 5. The ingestion rates of these amoebae

were 0.86–34.59 and 1.2–28.6 bacteria cell^{-1} h^{-1}, respectively, when fed with bacterial concentrations ranging from 4×10^6 to 3.5×10^8 cells L^{-1} (Table III). Heaton *et al.* (2001) refined the method of Butler and Rogerson (1997) by employing a GFP-expressing *Escherichia coli* as the prey bacterium, which avoided some problems encountered with the use of heat-killed stained bacteria (e.g., the loss of bacterial ability to encourage or deter ingestion by amoebae) (English, unpublished data). All the amoebae ingested naked, attached *E. coli*, though to different degrees (Table III). Heaton *et al.* (2001) also showed that when the *E. coli* cells were embedded in alginate (to mimic Prey form 3, Table II), three of the species ingested equivalent numbers of embedded *E. coli* cells to naked, recently attached cells (Table II), while the ingestion rate of *Vexillifera bacillipedes* was considerably reduced with embedded *E. coli* and *Saccamoeba limax* could not ingest this prey form at all. The reasons for reduced ingestion rates are not known at present, although one could hypothesize that in the case of the small *V. bacillipedes*, it may have been due to the alginate coating increasing the overall size of the *E. coli* particle. Another reason for the reduced/lack of ingestion might be that the alginate coating altered the ''taste'' of the prey.

F. Grazing Rates of Browsing Flagellates

The study by Carrias *et al.* (1995) suggested that browsing flagellates might include *Katablepharis ovalis* and the Bodonids; however, there is much evidence to show that *Bodo* spp. feed on suspended bacteria, with ingestion rates ranging from 2–250 bacteria cell^{-1} h^{-1} (Chrzanowski and Šimek, 1990; Daggett and Nerad, 1982; Davies and Sieburth, 1984; Eccleston-Parry and Leadbeater, 1994). Even so, *Bodo*—and other flagellates such as *Rhynchomonas, Cafeteria roenbergensis,* and *Caecitellus parvulus*—do appear to graze preferentially on attached bacteria (Caron, 1987; Sibbald and Albright, 1988; Zubkov and Sleigh, 2000). *Caecitellus parvulus* is an active flagellate adapted to surface feeding and has been found to consume 75% of *Vibrio natriegens* cells deposited onto a filter, yielding a specific growth rate of 0.82 day^{-1} and a GGE of 29% (Zubkov and Sleigh, 1999a). Unfortunately, no direct measurements of ingestion rate are available for browsing flagellates, but there are values for attached *Bodo saltans* and *Rhynchomonas* sp. feeding on suspended *Ps. putida* (i.e., 34.3 and 12.8 bacteria cell^{-1} h^{-1}, respectively) (Boenigk and Arndt, 2000a,b). In the latter study, *Rhynchomonas* was observed to feed on deposited prey cells, but only if they were loosely attached. Recent evidence suggests that flagellates are probably unable to graze on embedded bacteria. Mattison *et al.* (2002)

found that *Heteromita globosa* could control the growth of a slime-producing *Pseudomonas* sp. on a surface only if both prey and predator were co-inoculated at time zero. The flagellate had no effect on the bacterium when it was inoculated 60 days after the inoculation of the *Pseudomonas*, when a mucoid biofilm would have developed.

G. Grazing Rates of Browsing Ciliates

The study by Carrias *et al.* (1995) suggested that browsing ciliates might include *Strombilidium spp., Colpoda* sp., *Urotricha* sp., *Pseudobalanio planctonicum*, and *Prorodon* sp. Indeed, *Prorodon* has been shown to ingest DTAF-stained coliform bacteria at a rate of 169 bacteria $cell^{-1}$ h^{-1} in sediments (Epstein and Shiaris, 1992) (Table III). The ingestion rate of another browsing ciliate, *Chlamidodon* sp., was very similar, being 162 bacteria $cell^{-1}$ h^{-1} (Epstein and Shiaris, 1992). These values are within the range of 37–421 bacteria $cell^{-1}$ h^{-1} found by Kemp (1988) for *Euplotes* (with tintinnids) feeding in sediments. Ingestion rates of 3–176 FLB $cell^{-1}$ h^{-1} have been determined for *Euplotes* feeding on attached *Vibrio* spp. (Tso and Taghon, 1999), which is similar to the average rate of 254 bacteria $cell^{-1}$ h^{-1} determined for this genus feeding *on Ps. fluorescens* and *V. natriegens* attached to the surface of flow cells (Lawrence and Snyder, 1998) (Table III). The value determined by Albright *et al.* (1987) for *Euplotes* is much lower, 12.4 bacteria $cell^{-1}$ h^{-1}, even though 75% of the ciliate biomass came from these attached prey cells and similar attached prey concentrations were used in this study and that of Lawrence and Snyder, ($1-3 \times 10^5$ cells cm^{-2}) (Table III). The latter study was the first to determine surface clearance rates of *Euplotes*, being calculated as 0.03 and 0.02 mm^2 $ciliate^{-1}$ h^{-1} with *Ps. fluorescence* and *V. natriegens*, respectively (Lawrence and Snyder, 1998). These values are higher than the 0.0002–0.006 mm^2 $amoeba^{-1}$ h^{-1} determined by Heaton *et al.* (2001), suggesting that browsing ciliates may have a significant impact on attached bacterial cells.

H. Protozoan Grazing of the Biofilm Matrix

It is unclear whether the biofilm matrix is a source of nutrition for protozoa, as it has only recently been found to be biodegradable by bacteria (Zhang and Bishop, 2003). It is highly likely that some matrix will be ingested, indirectly, if embedded cells are grazed on by protozoa, but whether this is effectively digested is unknown. In general, the matrix is composed of a mixture of extracellular polysaccharides

(particularly alginate), proteins, nucleic acid, and phospholipids (Wingender *et al.*, 1999). Some heterotrophic flagellates can utilize high-molecular-weight materials such as dextrans, ferritin, casein, albumin, and Concavalin A (Marchant and Scott, 1993; Sherr, 1988; Tranvik *et al.*, 1993), but because it is unlikely that flagellates ingest embedded prey cells, the matrix might be unavailable to them. Joubert *et al.* (2003) have recently suggested that the browsing ciliate *Colpoda* can selectively feed on EPS excreted by yeasts, but more studies are required to corroborate this finding.

I. Summary of Protozoan Grazing on Biofilm-Associated Prey

In general then, sessile and transient ciliates should be able to consume all three forms of suspended prey (Prey forms 1, 5, and 6, Table II) together with recently attached prey (Prey form 2) because of sloughing processes. Sessile and transient flagellates should be able to consume all three suspended prey forms and recently attached cells (Prey forms 1, 2, 5, and 6) as long as the prey cell (with or without a capsule) is small enough. Amoebae should be able to consume recently attached and possibly embedded cells (Prey forms 2 and 3), but it is unknown whether they can consume deeply embedded cells. Finally, browsing ciliates and flagellates should be able to consume all three suspended prey forms and recently attached cells (Prey forms 1, 2, 5, and 6) with only the ciliates possibly consuming embedded cells (Prey form 3, and possibly 4).

IV. Nutrient Remineralization

Protozoa are known to play an important role in the remineralization of N and P within the plankton, but there have been no experiments to ascertain whether this role is important in biofilms or indeed planktonic aggregates. Protozoan remineralization is more effective in the plankton of oligotrophic environments because of the lack of allochthonous inputs of N and P. It is generally considered that attached algae (and possibly the other components) are limited at bulk water concentrations of 55 μg NL^{-1} and 1–5 μg PL^{-1} (Biggs and Close, 1989). However, the use of an optimal stoichiometric C:N:P ratio of 119:17:1 for biofilms has been proposed as a more appropriate indicator of the nutrient status of attached algae (Hillebrand and Sommer, 1999). If N:P ratios are <13, the attached algae are considered N-limited, while at N:P ratios >22, they are considered P-limited (Hillebrand and

Sommer, 1999). Unfortunately, data on cellular nutrient ratios is lacking, and most studies have investigated potential nutrient limitation in biofilms by using ''nutrient diffusing substrata'' (NDS) (Francoeur *et al.*, 1999; Lindstrom, 1996; Maberly *et al.*, 2002). Results have been variable, with authors concluding that attached algae are N-limited (Hillebrand and Sommer, 1997; Lindstrom, 1996; Tank and Dodds, 2003), P-limited (Matlock *et al.*, 1998; Notestein *et al.*, 2003) or limited by both N and P (Havens *et al.*, 1999; Maberly *et al.*, 2002). However, the variation in results might be related to the timings of the experiments, as nutrient limitation has been shown to vary seasonally (Francoeur *et al.*, 1999; Lindstrom, 1996).

Studies on the effect of the nutrients on the other components of biofilms are rare (Anderl *et al.*, 2003; Tank and Dodds, 2003), and because of this there is no general understanding of the role of protozoan remineralization within them. Protozoa are known to play an important role in the cycling of nutrients in soil (Clarholm, 1985), with the amoebae being responsible for 20–40% of the net N-remineralization (Davidson *et al.*, 1990). Thus it is plausible that protozoan remineralization could play a role in biofilms, but this has yet to be studied.

V. Grazing of Protozoa in Biofilms

A. Macro- and Mesoinvertebrate Grazers of Protozoa

Biofilms are an important food source for many stream invertebrates (Frost and Elser, 2002; Hillebrand and Kahlert, 2001; Lawrence *et al.*, 2002) and pelagic zooplankton (Jeppesen *et al.*, 2002). The grazers are either non-selective (e.g., mayfly) or selective (e.g., snails and ostracods, which graze the matrix and algae, but not the bacteria) (Lawrence *et al.*, 2002). Grazing can thus change the taxonomic composition of biofilms, whereas increased nutrient supply usually results in increased biomass of all biofilm components (Hillebrand and Kahlert, 2001). Experiments to determine the specific grazers of attached protozoa are rare, but biofilms contain a number of mesoinvertebrates such as rhabditoid nematodes (Martin-Cereceda *et al.*, 2001; McBain *et al.*, 2003). These are considered to be bacterivorous, and protozoa are known to outcompete them for bacterial prey in soil systems (Griffiths and Caul, 1993). Even so, nematodes have been shown to graze amoebae in soil (Elliot *et al.*, 1980; Woods *et al.*, 1982) and sessile ciliates, such as *Carchesium* and *Epistysis*, in freshwater biofilms (Kusuoka and Watanabe, 1989).

Other grazers of sessile ciliates include rotifers (e.g., *Eosphora anthadis*; Kusuoka and Watanabe, 1989). These are common components of biofilms (Green, 2003; Martin-Cereceda *et al.*, 2001; McBain *et al.*, 2003; Robbins *et al.*, 2000). In the plankton, a positive correlation between rotifers and protozoa (particularly ciliates) is common (Arndt, 1993) suggesting they are the major predators of protozoa (Gilbert and Jack, 1993). But Mohr and Adrian (2002) argue that ingestion of protozoa only enhances the survival of the rotifer, whereas reproduction is likely to rely on algal prey. Examination of foodwebs in an acid mine drainage system (pH 1.5) revealed the presence of only bacteria and heliozoans in the plankton, and bacteria, hypotrich ciliates, and bdelloid rotifers in the biofilm (Robbins *et al.*, 2000). This suggests that rotifers do not rely solely on algae but can feed effectively on browsing ciliates.

B. Intra-Protozoan Grazing

In addition to mesoinvertebrates grazing, predator-prey interactions will occur within the protozoan group itself. Ciliates are known to feed on flagellates and other ciliates (Bernard and Rassoulzadegan, 1991; Dolan and Coats, 1991; Epstein *et al.*, 1992; Laybourn-Parry, 1976; Stoeker and Evans, 1985). Less is known about the feeding preferences of amoebae, but they have been shown to feed on flagellates (Brussaard *et al.*, 1991) and ciliates (Gilbert *et al.*, 2003; Kusch, 1993b; Rogerson, 1980). Thus a complex level of predator-prey interactions in biofilms is expected, which would mirror that observed in the plankton (Arndt, 1993). However, it is envisaged that these interactions would be more intense in biofilms because of the close proximity of prey and predators. Such intensity should lead to defense mechanisms by which protozoa attempt to avoid predation, and this has indeed been recorded in the ciliate *Euplotes*, a common biofilm component. *E. octocarinatus* shows phenotypic plasticity in the presence of grazers, which induces it to form lateral projections and elongate the cell, making itself a more difficult particle to ingest (Kuhlmann and Heckmann, 1985). Recent evidence suggests that this is induced by direct contact between *Euplotes* and the predatory ciliate *Stylonychia mytilus* (Wiackowski and Starońska, 1999), but previous work has shown that it can be induced via chemical cues released by the predatory ciliate *Lembadion bullinum* (Kuhlmann and Heckmann, 1985), the tubellarian *Stenostomum sphagnetorum* (Kusch, 1993a), and the amoeba, *Amoeba proteus* (Kusch, 1993b).

VI. Cell Signalling in Protozoa

A. Signalling in Bacteria and Algae

Cell-cell signalling between bacteria in biofilms has been firmly established, with the signalling molecules being post-transcriptionally processed peptides in Gram-positive bacteria (Kleerebezem *et al.*, 1997) and N-acyl homoserine lactones (AHLs) in Gram-negative species (Greenberg, 1998). These highly diffusible molecules are secreted by the cells, and because their action is cell density-dependent, this signalling process has been termed ''quorum sensing.'' Quorum sensing is thought to play an important role in the development of biofilms (Davies *et al.*, 1998), and evidence for *in situ* production of AHLs has been reported (McLean *et al.*, 1997). Much of the communication is intraspecies specific, but inter-species communication (''cross-talk'') has also been found (Gray, 1997). Bacteria can communicate with algae, particularly those responsible for the biofouling of marine substrata. Attachment of the motile zoospores of *Enteromorpha* to a surface is known to be responsive to diffusible settling cues, which have mainly been bacterial in origin (Joint *et al.*, 2000). Some bacteria enhance zoospore settlement, while others inhibited their attachment (Thomas and Allsopp, 1983). Bacteria and diatoms can also produce diffusible chemical cues, which either stimulate or inhibit macroinvertebrate larval settlement (Harder *et al.*, 2002; Maki *et al.*, 1988).

B. Signalling in Protozoa

Mathematical models regarding the potential value of chemoreception in protozoa have suggested that even at high prey density, it would confer an advantage, while at low prey concentration it would be essential (Blackburn and Fenchel, 1999; Sibbald *et al.*, 1988). Early studies by Seravin and Orlovskaja (1977) showed that amoebae responded to inert particles coated with liver extracts by positive chemotaxis, while untreated particles were ignored. More recently, Matz *et al.* (2002) has shown that flagellates prefer to ingest microspheres coated with bovine serum albumin as compared with other protein- and-starch coatings.

Some prey cells are actively discriminated against for some chemical reason (Jürgens and De Mott, 1995; Landry *et al.*, 1991; Stoecker *et al.*, 1986). *Bacillus liqueniformis* can produce lytic compounds that prevent ingestion by amoebae (Harb and Kwaik, 2000), and the production of pigments by bacteria—for example prodigiosin (*Serratia marcescens*), pyocyanine (*Pseudomonas aerugenosa*) and violaceum

(*Chromobacterium violaceum*)—can also have an antibiotic effect on amoebae (Groscop and Brent, 1964). Other bacteria are positively selected for. Snyder (1991) showed that extracts from *Vibrio natriegens* and *V. neries* served as chemoattractants to the ciliate *Pseudocohnilembus marinus*, and *Euplotes* has been seen to gather in areas of high bacterial prey density and return to bare grazed areas possibly because of the persistence of a chemical cue from past feeding activities (Lawrence and Snyder, 1998). But *Euplotes* can also affect the behavior of its predators (e.g., *Litonotus lamella*, which is often seen gathering in areas of high *Euplotes crassus* abundance [Morelli *et al.*, 1999]). Cell free extracts of *E. crassus* have induced the same behavioral response in the predator, suggesting biological cues are involved (Morelli *et al.*, 1999). However, not all *Euplotes* species produce such cues. *E. magnicirratus* and *E. rariseta*, together with other ciliates such as *Aspidisca* sp., *Diophrys* sp., and *Euplotidium itio*, did not induce tracking by *Litonotus* (Ricki *et al.*, 1996).

Diffusible cues produced by protozoa can also induce changes in their bacterial prey such as ciliates inducing the formation of bacterial clusters or aggregates (Blackburn *et al.*, 1998; Hahn *et al.*, 2000) and *Acanthamoeba castellanii* enhancing the pathogenicity of six species of *Legionella* to human monocytes (Neumeister *et al.*, 2000).

VII. The Harboring of Pathogens by Protozoa

Freshwaters are subjected to anthropogenic inputs, which in addition to nutrients and pollutants will contain pathogenic organisms, particularly coliforms (Hunter and McDonald, 1991). In many circumstances, pathogenic bacteria have been detected in biofilms when their presence in the bulk water has been undetectable (English *et al.*, 2001; Szewzyk *et al.*, 1994). Thus the bacterial community of freshwater biofilms may have a higher proportion of pathogenic strains within it compared with the plankton, and this will undoubtedly affect their major grazers, the protozoa.

The interaction between bacterial pathogens and protozoa has received considerable attention since Barker and Brown (1994) nicknamed protozoa the ''Trojan Horses of the Microbial World'' because certain pathogenic bacteria can survive and replicate within them. To survive within the intracellular environment of a protozoan host, the bacteria must avoid the natural killing mechanisms of the host (food vacuoles) and be able to use the nutritional reserves of the host cell (Harb and Kwait, 1999). Some bacteria have adapted to the low pH environment of the food vacuole (e.g., *Coxiella burnetii*), while others

have exploited this acidification to aid escape into the cytoplasm (e.g., *Listeria monocytogenes*) (LaScola and Raoult, 2001). Other bacteria prevent the acidification of the food vacuole altogether by excluding the ATPase proton pump from the host vacuolar membrane (e.g., *Mycobacterium avium*) (Sturgill-Koszycki *et al.*, 1994). Once the host killing mechanism has been evaded, the fate of the interaction falls into one of three categories: those that replicate and lyse the host; those that replicate but do not lyse the host; and those that neither replicate nor lyse the host but are egested as whole, viable cells. Some members of the coliform group have been shown to comprise the latter group, *Escherichia coli* in particular (Schlimme *et al.*, 1997). The egested bacteria often possess different physiological features from their undigested counterparts—typically, an increased resistance to antimicrobial compounds (Barker *et al.*, 1995). This is thought to be due to the bacteria exhibiting the general stress response (GSR) in the food vacuole, which involves a stationary-phase cascade during which cells become quiescent (Brown and Barker, 1999). The genotype of some egested bacteria has also changed after *passage* through protozoa. Schlimme *et al.* (1997) showed how the food vacuoles of *Tetrahymena pyriformis* were ''hot spots'' for horizontal gene transfer, with significantly increased levels of conjugation between strains of *E. coli*.

Of those bacteria that replicate inside the protozoan host, some do not lyse the host cell (e.g., *Vibrio cholera, E. coli* O157) (Barker *et al.*, 1999), while others do (e.g., *Legionella pneumophila* and *L. monocytogenes*) (Fields *et al.*, 1984; Rowbotham, 1980). It is currently estimated that *L. pneumophila* can survive and replicate in 2 species of the ciliate *Tetrahymena* and in 13 species of amoebae, which include *Acanthamoeba* (5 species), *Naegleria* (4 species), *Hartmannella* (2 species), *Echinamoeba*, and *Vahlkampfia* (Murga *et al.*, 2001). The interaction has been shown to be temperature dependent, whereby intracellular replication occurs at temperatures of 35 °C, but at temperatures of $<$20 °C, which are more environmentally relevant, the bacterial cells are phagocytosed and digested (Anand *et al.*, 1983). The extent to which such parasitic interactions occur in nature is unclear, but *L. pneumophila* has been shown to survive within amoebic cysts (Kilvington and Price, 1990), indicating that it is not only the active trophozoites that have a role in harbouring pathogens.

The interaction between bacterial pathogens and protozoa is interesting not only from an ecophysiological point of view but also from an evolutionary point of view. It has been hypothesized that pathogenic bacteria may have developed as such by ''practicing'' evading protozoan digestion in the environment, thereby allowing them to evade

digestion by macrophages in the immune system (Brown and Barker, 1999). Some of the cellular mechanisms seen when amoebae and human macrophages are infected with *L. pneumophila* are similar (Barker *et al.*, 1995; Newsome *et al.*, 1998), and it has been shown that the pathogen utilizes the same genes to multiply within the two types of phagocytic cell (Segal and Shuman, 1999). Amoebae are now being used as surrogate macrophages in laboratories around the world.

VIII. Conclusions and Future Prospects

Protozoan participation in the formation and maintenance of freshwater biofilms has received little attention even though it has been estimated that 99% of microbial activity is associated with surfaces. Most studies have been qualitative in nature, and from these it is known that many species of protozoa exist on surfaces at concentrations higher than those in the bulk water. Only a handful of studies have attempted to quantify the grazing impact of these biofilm-associated protozoa but a picture is now starting to develop, though it is far from complete. Further research is necessary, with comparable experimental techniques, to deduce the nature of these interactions, and their significance on the biogeochemical cycling of nutrients and harbouring of pathogens in freshwater systems.

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Metals in Yeast Fermentation Processes

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I. Introduction

Yeast cells have been used for millennia in traditional fermentations of cereal mashes, grape musts, and other naturally derived substrates. These processes still represent very important industries pertinent to the brewing, baking, winemaking, and distilling sectors. The substrates in question provide rich sources of fermentable carbohydrate, utilizable nitrogen, vitamins, other growth factors, and minerals. Unfortunately, the latter are often overlooked as important determinants of yeast fermentation performance (see Fig. 1), and it should be emphasized from the outset that the nature and concentration of metal ions

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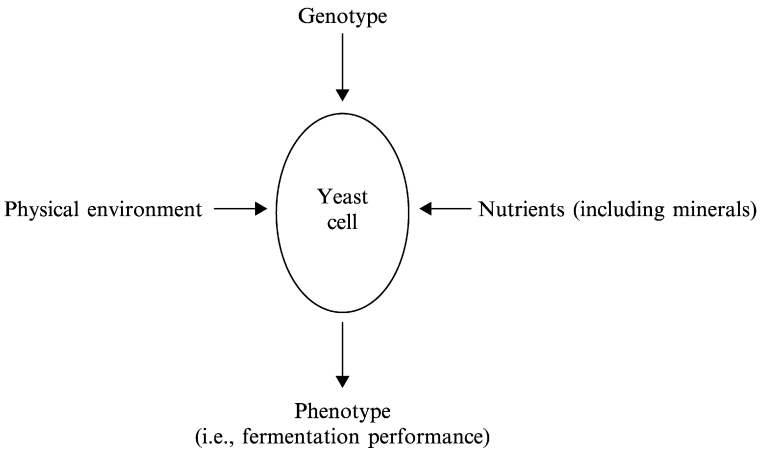


FIG. 1. Factors affecting yeast fermentation performance.

supplied in growth media can have a significant impact on yeast-based industrial processes. After all, a prerequisite for the success of any yeast biotechnology is a thorough understanding of the factors that regulate nutrition, growth, stress responses, and metabolism in yeast cells. These include inorganic factors.

Yeast cells require a wide range of metals for their growth and metabolic functions, and the mineral nutrition of yeasts is thus very important in ensuring successful fermentation, particularly in alcohol production processes. The *bioavailability* of essential metal ions in industrial media can dramatically influence yeast fermentation performance. For ethanol fermentations, these ions include magnesium and zinc that act as co-factors for important fermentative enzymes and also as modulators of environmental stress. Some metals inhibit yeast growth and metabolism, either by antagonism with essential metals (for example, calcium against magnesium) or through direct toxicity effects (as with heavy metals). This chapter reviews the mineral nutrition of yeasts employed in fermentation processes, with a particular focus on the roles of magnesium, calcium, and zinc in the physiology of industrial strains of the yeast *Saccharomyces cerevisiae*.

II. Overview of Yeast Fermentation Processes

The so-called ''conventional'' yeast, *S. cerevisiae*, represents the most exploited microbe known to mankind, being responsible for the production of many diverse commodities from beer to blood

proteins. Following developments in recombinant DNA technology, *S. cerevisiae* is now widely employed to express foreign genes and synthesize a range of health-care proteins including hormones, serum albumin, enzymes, vaccines, and other pharmaceuticals. Table I provides an overview of some yeast products important in modern biotechnology.

In recent years it has become increasingly apparent that *S. cerevisiae* may not be the best yeast species to use in the production of high-value biopharmaceuticals. Other ''non-conventional'' yeasts—notably *Schizosaccharomyces pombe, Kluyveromyces lactis, Pichia pastoris, Hansenula polymorpha*, and *Yarrowia lipolytica*—display distinct advantages over *S. cerevisiae* in expression and secretion of human therapeutic proteins and enzymes (Wolf *et al.*, 2003).

Unfortunately, our knowledge of the cell physiology of non-*Saccharomyces* yeasts is still rudimentary, and this also refers to mineral nutrition aspects.

TABLE I

DIVERSITY OF SOME YEAST FERMENTATION PRODUCTS

Yeast species	Examples of industrial fermentation products
Saccharomyces cerevisiae	Beer, wine, distilled spirits, bioethanol, baked foods, probiotics/animal food supplement, organic chemical reductions, hepatitis B vaccine, human insulin, human serum albumin
Schizosaccharomyces pombe	Some bioethanol, rum, wine-deacidification, indigenous fermented beverages, recombinant proteins
Kluyveromyces spp.	Cheese whey fermentations, biomass protein, pectinases, recombinant chymosin
Pichia pastoris	Recombinant proteins
Hansenula polymorpha	Recombinant proteins
Yarrowia lipolytica	Recombinant proteins
Phaffia rhodozyma	Food and feed pigment (astxanthin)
Candida utilis	Biomass protein
Zygosaccharomyces rouxii	Traditional oriental fermented food (e.g., soy sauce, miso)

III. Nutrition of Yeasts Employed in Fermentation Processes

A. Yeast Growth v. Fermentation

The primary aim of a yeast cell is to produce more yeast cells. This is also the case during industrial fermentation processes, and the metabolites secreted during yeast growth merely represent waste products as cells strive to maintain their redox balance. Many of these metabolites are valuable fermentation products, an important example being ethanol, which is produced when cells regenerate NAD in an attempt to keep glycolysis going and to make sufficient ATP for cellular biosyntheses. Ethanol cannot be produced without significant yeast cell growth, and non-growing yeast cells ferment only enough sugar to produce energy for cell maintenance. Therefore, the dilemma facing distillers, brewers, and winemakers is one of supplying sufficient nutrients to yeast to carry out fermentation while minimizing yeast growth. For industrial alcohol producers, excess yeast represents alcohol loss, but it has been calculated (Ingledew, 1999) that growing cells produce alcohol 33 times faster than non-growing cells! Compromise efforts are made to keep yeast under conditions that do not lead to low growth rates or to cell death. Minimizing yeast growth during alcoholic fermentation may be accomplished by employing high yeast cell densities/cell re-cycle systems, continuous/semi-continuous fermentations, or immobilized yeast bioreactors. In addition, it would be desirable to encourage a predominantly fermentative, rather than respiratory, mode of metabolism in the yeast strains employed for alcohol production. Metal ions may play a role in this metabolic regulation. For example, Walker *et al.* (1982) have shown that the availability of magnesium ions can dictate whether fermentation or respiration predominates under certain conditions of yeast cultivation. This concept is discussed further by Walker (1994), who has proposed that under respirofermentative conditions, magnesium governs the flow of carbon into fermentation or respiration based on the relative affinities of pyruvate metabolizing enzymes for intracellular free magnesium ions.

B. Carbon and Nitrogen Requirements

Walker (1999a) has reviewed industrial growth media commonly employed in traditional yeast fermentation processes for the production of foods and beverages. Being chemoorganotrophs, yeasts require organic substrates as carbon and energy sources. Most yeasts employed in industrial fermentations, namely strains of *S. cerevisiae*, effectively utilize sugars such as sucrose, glucose, fructose, and maltose for their

growth and metabolism. Sources of these sugars are extracted from sugar crops (cane and beet juice and molasses), fruit juices (wine must), and cereal starches (barley, maize, and wheat starch hydroylsates). Non-*Saccharomyces* yeasts can extend the range of carbon sources for industrial processes, and these include lactose (fermented by *Kluyveromyces marxianus*), xylose (*Pichia stipitis, Candida shehatae*), methanol (*Pichia pastoris, Hansenula polymorpha*), starch (*Schwanniomyces occidentalis*), inulin (*Kluyveromyces marxianus*), and *n*-alkanes (*Yarrowia lipolytica*). Table II summarizes the diversity of carbon sources available to yeasts for industrial fermentation processes.

In terms of nitrogen sources, many of the plant-based fermentation media listed in Table II also provide yeasts with readily utilizable sources of nitrogen essential for cellular biosyntheses and enzyme/nucleic acid function. *S. cerevisiae* is non-diazotrophic (cannot fix nitrogen) and non-proteolytic (being unable to utilize proteins as nitrogen sources). Various types of hydrolyzed proteins—for example, corn steep liquor, casein, soybean, barley malt, and yeast extract—provide mixtures of amino acids and small peptides that are able to support *S. cerevisiae* growth during fermentation. Additional forms of inorganic nitrogen, such as ammonium salts and urea, may be required as supplements for some natural complex yeast media. For distillery yeasts, levels of ammonium ions, urea, and free alpha-amino nitrogen (FAN) are assimilable, but can be growth limiting. Ingledew (1999) has reported that the growth of distilling strains of *S. cerevisiae* increases almost linearly with FAN levels up to 100 mg/L. Some types of molasses may be deficient in assimilable nitrogen (e.g., total N-compounds are only 2–3% in cane molasses) and must be supplemented with ammonia or urea (Walker, 1999a).

Many of the agriculturally derived yeast media are considered complete in that they supply not just rich sources of carbon and nitrogen but also a range of other nutrients including vitamins and minerals. Mineral requirements of yeasts will now be addressed with the following discussions, focusing primarily on *S. cerevisiae* alcoholic fermentation processes.

C. Mineral Requirements

1. *Why Do Yeasts Need Metals?*

Metals are very important in several areas of yeast cell physiology. For example, yeast cells need metals for maintaining cell and organelle structural integrity, for cell–cell interactions such as flocculation, for gene expression, for cell division and growth, for nutrient uptake

TABLE II

CARBON SOURCES FOR MAJOR YEAST FERMENTATION PROCESSES

Carbon form	Examples	Industrial source	Yeasts involved	Products
Hexose sugars	Glucose, fructose	Grape juice	*S. cerevisiae*	Wine
	Glucose	Starch hydrolysates	*S. cerevisiae* and other yeasts	Recombinant proteins, pharmaceuticals
Pentose sugars	Xylose, arabinose	Wood/cellulosic hydrolysates, corn steep loquor	*Pichia stipitis, Candida shehatae*	Ethanol, biomass
Disaccharides	Sucrose	Sugar cane/beet juice and molasses	*S. cerevisiae*	Ethanol, baker's yeast, food extracts
	Maltose	Cereal mashes	*S. cerevisiae*	Beer, distilled spirits
	Lactose	Cheese whey	*Kluyveromyces marxianus*	Ethanol, biomass
Polysaccharides	Starch	Cereals, tubers	*Schwanniomyces*	Ethanol, biomass
	Insulin	Tubers (Agave, artichoke)	*Kluyveromyces*	Ethanol, biomass, enzymes
Aliphatic alcohols	Ethanol	Distilling residues	*Candida utilis*	Biomass protein
	Methanol	Petrochemicals	*Pichia pastoris, Hansenula polymorpha*	Recombinant proteins
Hydrocarbons	C_{12}-C_{18} *n*-alkanes	Petrochemicals	*Yarrowia lipolytica*	Biomass, recombinant proteins

mechanisms, for enzyme action in metabolism, for osmoregulation, and for energy maintenance and cell survival. Additionally, yeast cells need metals as stress-protectants in the face of environmental insults (refer to Section VI.E).

Bulk metals, such as potassium and magnesium, are generally required by growing yeast cells in the millimolar concentration range, and the trace metals such as calcium, manganese, zinc, iron, and copper are required in the micromolar range. These essential metals play numerous structural and functional roles in yeast cell physiology. Other metals, even at trace level concentrations, may be toxic to yeast and these include heavy metals (see below). Jones and Gadd (1990) have reviewed yeast inorganic nutrition.

2. *Essential Metals for Yeast Growth and Metabolism*

In general terms, metal ions can impact on yeast growth and metabolic processes during fermentation by influencing several important parameters. For alcohol fermentations, these include the rate of sugar conversion to ethanol, the degree of attenuation/final ethanol yield, the amount of yeast produced, cell viability and stress tolerance, extent of foaming, and yeast flocculation behavior. All of these parameters can impact significantly on the efficiency of industrial yeast fermentations. Table III lists those metals and their approximate concentrations generally required for cellular growth and reproduction of *S. cerevisiae*. The figures quoted are approximate because precise metal requirements will differ depending on the particular strain of yeast and the cultivation conditions.

Potassium, magnesium, calcium, and zinc are cationic nutrients that play essential structural and functional roles in yeast cells and are particularly significant in fermentation processes. Potassium is the most abundant cellular cation in yeast, constituting 1–2% of yeast cell dry weight, and is the main electrolyte essential for osmoregulation, charge-balancing of macromolecules, and regulation of phosphate and divalent cation uptake (Jones and Greenfield, 1994). Potassium additionally acts as a major cofactor for enzymes involved in oxidative phosphorylation, protein biosynthesis, and carbohydrate catabolism.

Sodium is the other main monovalent cation, but it is important to note that although yeast cells may sometimes contain quite high levels of sodium, and fermentation media are also often high in sodium, this metal appears to be non-essential for yeast. For example, under normal growth conditions, *S. cerevisiae* actively excretes sodium (via a sodium-proton antiporter) to maintain intracellular sodium at very low, sub-toxic levels. Although certain halotolerant and marine yeasts

TABLE III

METALS REQUIRED FOR YEAST CELL GROWTH AND METABOLIC FUNCTIONS

Metal ion	Concentration supplied in growth medium*	Main cellular functions
Macroelements		
K	2–4 mM	Osmoregulation, enzyme activity
Mg	2–4 mM	Enzyme activity, cell division
Microelements		
Mn	2–4 μM	Enzyme cofactor
Ca	$<\mu$M[†]	Second messenger, yeast flocculation
Cu	1.5 μM	Redox pigments
Fe	1–3 μM	Haem-proteins, cytochromes
Zn	4–8 μM	Enzyme activity, protein structure
Ni	$\sim$10 μM	Urease activity
Mo	1.5 μM	Nitrate metabolism, vitamin B12
Co	0.1 μM	Cobalamin, coenzymes

*Figures relate to *S. cerevisiae* growth stimulation, but are dependent on the yeast species/strain and precise conditions of growth.

[†]See text for further discussion on calcium requirements for yeast growth.

(e.g., *Debaryomyces hansenii*) grow well in saline environments, there is no evidence to suggest that *S. cerevisiae* needs sodium for cellular growth, even at very low concentrations. If sodium is present at high concentrations it may prove toxic to yeast, possibly by antagonizing essential potassium-dependent functions.

Magnesium is the most abundant intracellular divalent cation in all living cells and is absolutely essential for yeast growth. Magnesium-deficient cells will not complete mitosis, and no other metal in the periodic table can substitute for magnesium in this role (reviewed by Walker, 1994). Magnesium constitutes around 0.3% of yeast cell dry weight and acts as an essential cofactor for over 300 enzymes intimately involved in many metabolic and bioenergetic pathways (e.g., magnesium is an absolute requirement for the synthesis of DNA and ATP). Changes in intracellular magnesium concentration can dramatically influence enzyme activity, and Grubbs and Maguire (1987) have proposed a key regulatory function for magnesium ions in eukaryotic cell metabolism. Together with potassium, magnesium can neutralize the electrostatic forces in nucleic acids, polyphosphates, and proteins. Concerning the

latter, magnesium maintains the tertiary structure of proteins and the general structural integrity of cells and organelles. Magnesium can also shield charged phospholipids and in doing so can maintain the structure of membranes, especially when cells are stressed. In short, magnesium plays multifaceted roles in yeast cell physiology at the cytological, biochemical, and biophysical levels. Importantly with regard to industrial fermentation processes, magnesium is necessary for the activation of several glycolytic enzymes (e.g., all those involved in transfer of phosphate moieties). In practical terms, this means that if industrial medium is magnesium-limited, the conversion of sugar to alcohol may be suppressed leading to slow or incomplete fermentation processes.

Calcium has long been ascribed a pivotal role as a second messenger of external stimuli in eukaryotic cells. Minute changes in intracellular calcium trigger cascades of protein kinase activity, leading ultimately to initiation of key events such as the onset of mitosis. However, these changes in calcium are extremely small, and levels of intracellular free calcium are maintained at very low (sub-micromolar) levels. This, in turn, means that calcium requirements for cell division and growth are also very low. For yeast growth, we should therefore consider calcium to be a trace metal. Calcium binds to yeast cell walls and plays a key role in flocculation, which is important in brewing fermentations. Calcium also antagonises uptake of magnesium and can block essential magnesium dependent metabolic processes. Calcium-magnesium antagonism, especially as it relates to yeast fermentation processes, is discussed further below.

As for other trace elements, iron, zinc, nickel, copper, cobalt, manganese, and molybdenum are required in metalloenzymes, redox pigments, haem-proteins, and vitamins as structural stabilizers and as essential cofactors. For enzymes, some of these metals bind to catalytic active sites, and this is the case with zinc. In alcoholic fermentations, zinc is particularly important with regard to its role as activator of the terminal alcohologenic Zn-metalloenzyme ethanol dehydrogenase. Media deficient in zinc may lead to slow or incomplete fermentations, and this has long been recognized as an occasional problem in the brewing industry (as discussed below).

IV. Interaction of Yeasts with Metals

A. Mineral Contents of Yeast Cells

Table IV shows the mineral content of a ''typical'' yeast cell. As with Table III, the figures quoted are approximations, because precise values of cellular minerals will depend on the particular yeast strain in

TABLE IV

AVERAGE ELEMENTAL COMPOSITION OF *SACCHAROMYCES* (G/KG DRY WT)

Potassium 22	Phosphorus 16	Sulphur 3
Magnesium 2.7	Sodium 0.6	Calcium 0.5
Barium 0.15	Zinc 0.12	Iron 0.1
Copper 0.05	Manganese 0.03	Cobalt 0.005
Nickel 0.0025	Arsenic 0.0018	Lead 0.0015
Iodine 0.00125	Molybdenum 0.0007	Boron 0.0005
Aluminium 0.0001	Chromium 10×10^{-25}	Vanadium 5×10^{-25}

question and its growth conditions. In addition, the phase of yeast growth and the position of cells in the cell division cycle may result in different cellular metal concentrations. For example, Walker and Duffus (1980) have shown that the magnesium content of dividing yeast cells varied in a temporal manner with cell cycle progress. For the fission yeast, *Schizosaccharomyces pombe*, it was revealed that intracellular magnesium levels fell during growth until a point just prior to mitosis, when a large influx of magnesium took place. This ensured that daughter cells at cell division received the same magnesium content as their mother cells had originally at the start of their cell cycle. Magnesium influx just before cell division was proposed to govern the disassembly of the mitotic spindle, specifically by de-polymerisation of tubulin, the major structural protein of microtubules. Walker (1986) has further discussed this role of magnesium in cell cycle control. The metal content of yeast cells also depends on the phase of growth during cultivation in liquid medium and will vary between lag, logarithmic, and stationary phases of the batch growth cycle. Walker and Duffus (1980) have shown changes in yeast cell magnesium levels during transitions between the lag and logarithmic growth phases and Walker and Maynard (1997) showed *that S. cerevisiae* cells released magnesium at the onset of the stationary phase.

Yeasts display differential affinities for certain metal ions. For example, *S. cerevisiae, Schizo. pombe,* and *Candida utilis* possess high growth affinities for magnesium, with respective Ks (saturation coefficients) values of 36 μM (Walker and Maynard, 1996), 20 μM (Walker *et al.*, 1990), and 15 μM (Shkidchenko, 1977). These micromolar values reflect the high growth demands that yeasts have for magnesium. This means that it is feasible to prepare Mg-limited growth media for yeast and to grow cells under Mg-limited conditions in a chemostat. Walker and Maynard (1996) accomplished this for *S. cerevisiae* and were able

to facilitate studies of yeast cell physiology under conditions in which cell growth was dictated solely by magnesium ion availability. Such experiments would not be feasible with calcium because yeasts have a low growth demand for this metal (i.e., high Ks value), and it is not possible to cultivate cells in a chemostat with calcium as the sole growth-limiting nutrient.

B. Yeast Transport Strategies for Metals

Yeast cells can transport, localize, compartmentalize, and sequester metals required for various physiological functions and can neutralize metals that are potentially toxic. These functions include intracellular pH homeostasis, osmoregulation, enzyme function, protein structure, membrane stabilization, and signal transduction. For yeast growth and survival, cellular concentrations of metals are maintained within relatively narrow ranges though a variety of homeostatic mechanisms (reviewed by Walker, 1998a). To take up metals from their growth environment, yeast cells must transport metals as free, ionized forms. Therefore, several physico-chemical constraints may impede metal ion uptake by yeast, and these include chelation, adsorption, and binding. In complex growth media such as sugarcane molasses or malt wort, this can lead to reduced metal bioavailability during fermentation. To increase metal bioavailability from their growth environment, some yeast species may mobilize metal ions by secreting low-molecular weight metal-sequestering compounds called *siderophores* (Van der Helm and Winkelmann, 1994) or organic acids such as citric acid (White *et al.*, 1997). Although a few yeasts have been shown to excrete siderophores (for iron uptake), yeasts more commonly internalize essential metals through specific membrane transport systems. However, to be transported into the yeast cellular milieu, several barriers first need to be overcome by metals. These include the capsule (exopolysaccharide layer, if present), cell wall, periplasm, plasma membrane, and organellar membranes. Specific transport mechanisms employed by yeast depend on the bioavailability of metal ions and the prevailing environmental conditions, but generally, most metals bind to yeast cells in a biphasic manner: first by non-specific cell surface biosorption and second by selective transmembrane-mediated translocation into the cytosol. To facilitate the latter, the following strategies may be adopted: free diffusion, facilitated diffusion, diffusion channels, and active transport. Of these, the latter two are most likely to operate in *S. cerevisiae* with a proton-pumping ATPase-mediated mechanism prevailing for the majority of metal ions. This enzyme is very important

for metal accumulation in yeast, but it also regulates growth and fermentation by excreting acidity and regulating cell pH (yeasts can lower external pH to ~1.5 and during fermentation, and around 30% of media acidity is attributed to ATPase activity). The primary driving force for the ATPase-mediated mode of metal uptake by yeast is the membrane potential and the transmembrane electrochemical proton gradient, generated by ATP-hydrolase activity. The latter extrudes protons by using the free energy of ATP hydrolysis and enables metal ions to enter yeast cells either with influxed protons (as in symport mechanisms) or against effluxed protons (as in antiport mechanisms). Such a mechanism requires participation of metal-translocating permeases, of which there are several specific high-affinity types identified in *S. cerevisiae*. These processes can also operate in the opposite direction to facilitate controlled efflux of metals (e.g., calcium and copper) to maintain low intracellular levels. Some low-affinity, relatively non-specific, permeases operate to transport metals when they are present in high abundance extracellularly.

The other main mechanism for metal uptake by yeast cells involves diffusion channels that are voltage-dependent membrane proteins activated by membrane depolarisation to influx (or efflux) specific ions like potassium (Reid *et al.*, 1996). In fermenting cells of *S. cerevisiae*, potassium accumulation is rapid. Mechanosensitive ion channels also exist in yeast cell membranes to control calcium ion homeostasis (Gustin *et al.*, 1986).

C. Molecular Biology of Metal Uptake by Yeast

As discussed above, the majority of metals are taken up into yeast cells via specific active transport proteins. These transporters possess varying affinities for particular metals: high-affinity systems ensure that essential metals are accumulated under conditions of limited availability, while low-affinity systems control uptake when metals are present in excess. Recent molecular genetic studies with *S. cerevisiae* have revealed several genes encoding specific metal ion transporters (see Table V).

Eide (1998) has reviewed the molecular biology of metal transport in yeast, and some of the *S. cerevisiae* genes have now been shown to encode proteins capable of transporting several metals. For example, the Smf proteins (encoded by the *SMF* family of transport genes) play a major role in regulating copper and manganese homeostasis and, under certain conditions, Smf1p may also function in iron assimilation by cells (Cohen *et al.*, 2001). There are many similarities between

TABLE V

SOME METAL ION TRANSPORTERS IN *S. CEREVISIAE*

Genes	Transporters	Comments
ZRT1/2, ZRT3	Zn	High/low affinity; vacuolar
IRT 1, FET, FTR, FRE	Fe	Also transports Mn and Zn
SMF1/2, CDC1, PMR1, CCC1, ATX1	Mn	Membrane, cytosol, Golgi transporters
CTR1, CCC2	Cu	Cellular and Golgi transporters
ALR1/2, MRS2	Mg	Membrane and mitochondrial
Channel protein-encoding gene	Ca	Mechanosensitive ion channel

human and yeast cells in terms of metal uptake mechanisms and genetic homology in *Saccharomyces cerevisiae* and *Homo sapiens* has now been demonstrated for Fe, Cu, Mn, and Mg transporters (e.g., Zsurka *et al.*, 2001). This has led to yeast being used to study the molecular bases of certain human genetic disorders linked to dysfunction of metal homeostasis, including Wilson's and Menkes syndromes. These are diseases of copper overload and copper deficiency, respectively. Remarkably, the relevant human genes that regulate copper homeostasis can substitute for their *S. cerevisiae* counterparts, enabling their structure and function to be effectively studied in yeast (Askwith and Kaplan, 1998; Nelson, 1999).

For cellular magnesium transport by *S. cerevisiae*, two plasma membrane transporters, encoded by *ALR1,2* genes and one mitochondrial transporter, encoded by the *MRS2* gene, have been demonstrated. The latter shows homology with a human mitochondrial Mg transporter. Recent evidence (Lui *et al.*, 2002) has been presented that implicates Alr1p as a Mg-channel transporter in yeast cells. *ALR1* encodes a 96 kDa membrane-spanning protein that transports Mg, and mutants lacking *ALR1* contain much less Mg and need high Mg levels for growth (Graschopf *et al.*, 2001). MacDiarmid and Gardner (1998) have also shown that *ALR1* increases tolerance to Al^{3+} in yeast and that inhibition of magnesium uptake may be the main cause of aluminium toxicity in yeast. In acidic soil conditions, aluminium can be leached from insoluble forms and in certain industrial fermentation processes, notably those employing sugar cane molasses, aluminium may be toxic to yeast. Suppression of yeast fermentation performance by aluminium may possibly be ameliorated by magnesium.

D. Fate of Intracellular Metals in Yeast

Once transported into yeast cells, metals may end up in different cellular locations, including the following: free in cytoplasm at very low concentrations (often sub-μM); sequestration in cytoplasm (by metallothioneins, calmodullin, polyphosphates and polyamines); compartmentalization (in the cell wall, vacuole, Golgi apparatus, mitochondrion and nucleus), or detoxification/transformation (following reduction, methylation and dealkylation). Considering compartmentalization of metals in yeast cells, selective transport protein genes have now been identified that control organellar membrane transport. For example, in *S. cerevisiae* the *CCC2* encoded protein regulates export of copper into the lumen of the Golgi; the *PMR1* gene is involved with uptake and release of manganese from vacuoles; the *MRS* gene controls mitochondrial uptake of magnesium and the *ZRT3* gene mediates zinc uptake in the vacuole. The yeast vacuolar membrane, called the tonoplast, is thought to play an important role in regulating ionic homeostasis and in detoxification of potentially toxic metals in yeast. Tonoplast uptake mechanisms resemble those of the yeast plasma membrane with proton-pumping ATPases involved in transport of magnesium, manganese, iron, zinc, cobalt, calcium, and nickel to the yeast vacuole. Beeler *et al.* (1997) have shown that, in *S. cerevisiae*, the vacuole plays an important role in regulating intracellular magnesium levels, especially under magnesium-limited growth conditions. Similarly, MacDiarmid *et al.* (2000) have shown that the vacuole plays a key role in regulating zinc homeostasis in yeast.

The cell wall is also a major site for metal localization in yeast, and this mode of metal binding is often referred to as *biosorption* or *bioaccumulation* (Brady and Duncan, 1994; Engl and Kunz, 1995; Fuhrmann and Rothstein, 1974; Norris and Kelly, 1977; Walker, 1985). This represents a biophysical attachment of metals to negatively charged cell wall moieties (e.g., carboxyl groups), and is the first step in the biphasic uptake of metals by yeast (the second being transmembrane uptake). Metal binding to yeast cell walls is an immediate, fairly non-specific event. With regard to yeast fermentation processes, the cell wall binding of calcium ions is important in flocculation mechanisms. This phenomenon is particularly relevant for brewing strains of *S. cerevisiae*. Calcium is thought to participate in yeast flocculation by activating cell wall α-mannan residues, thus enabling lectin proteins to facilitate adhesion between adjacent yeast cells (Miki *et al.*, 1982). Another yeast cell–cell interaction phenomenon which involves metal ions is agglomeration. This is also called yeast ''grittyness'' and is occasionally

experienced following the growth of baker's yeast (*S. cerevisiae*) on molasses. Agglomeration is detrimental to yeast quality for baking because cells fail to resuspend in water, and this adversely affects subsequent fermentation performance. Although it is a type of yeast cell adhesion, agglomeration is distinct from flocculation (which is a reversible process). Guinard and Lewis (1993) have proposed that calcium ions were involved in promoting baker's yeast agglomeration, while more recently, Birch *et al.* (2002) have shown that magnesium acted antagonistically against calcium-induced agglomeration, possibly by blocking calcium binding to cell surface receptors.

E. Metals Toxic to Yeast and Detoxification Strategies

Many metals are toxic to yeast cells, but the degree of toxicity depends on the actual metal in question, its concentration, and its bioavailability. Heavy metals generally adversely affect yeast growth at concentrations greater than around 100 μM (Rose, 1976). Metal-induced toxicity toward yeast is expressed at the levels of both cytotoxicity and genotoxicty through damage inflicted on cellular proteins and DNA, respectively. Metals that may occasionally prove toxic to yeast during fermentation processes include copper, cobalt, aluminium, manganese, cadmium, zinc, nickel, mercury, arsenic, and lead. For example, copper is an essential metal for yeast respiratory pigments, but above certain threshold concentrations it may be toxic. Some yeasts, together with filamentous fungi, have the ability to carry out heavy metal detoxification by using a variety of strategies including chemical transformation, sequestration, cell wall biosorption, immobilization, and protection (e.g., binding competition or membrane stabilization by beneficial metals). Potentially toxic levels of calcium ions are maintained at very low levels (often sub-micromolar) by intracellular binding to Ca-specific proteins such as calmodulin, which has been identified in *S. cerevisiae* and other yeasts. In certain yeasts and in filamentous fungi, intracellular sequestration of metals may also be achieved by binding to metallothioneins and phytochelatins (Winkelmann and Winge, 1994). Metallothioneins are small cysteine-rich polypeptides that bind to essential metals such as copper and zinc, as well as to toxic metals such as cadmium. Copper-resistance in *S. cerevisiae* is conferred by induction of copper-metallothionein biosynthesis. Phytochelatins are D-glutamyl peptides derived from glutathione that are involved in heavy metal detoxification in some yeasts and fungi, as well as in plants and animals. Yeasts can also

chemically transform metals to reduce their toxic effects (see Gadd and Sayer, 2000). Such transformations involve reduction (e.g., Cu [II] to Cu [I]; Fe [III] to Fe [II]; and Se [VI] to Se [IV] to elemental Se), methylation (e.g., of arsenic and selenium), and dealkylation (e.g., of organotin compounds to Sn [II] and organomercury compounds to Hg).

Magnesium has been shown to alleviate the toxic effects of several heavy metals, including aluminium (McDiarmid and Gardner, 1996), cadmium (Kessels *et al.*, 1985), cobalt (Aoyama *et al.*, 1986), copper (Karamushka and Gadd, 1994), manganese (Blackwell *et al.*, 1997), and zinc (Karamushka *et al.*, 1996). These protective effects of magnesium are thought to be mediated by membrane stabiliziation (e.g., charge neutralization of phospholipids) and competitive membrane binding in the face of heavy metal toxicity. This may have some practical implications for yeast fermentation processes (see below).

V. Practical Significance of Metal Uptake by Yeast

A. Bioremediation

This relates to removal of heavy metals in industrial wastewaters, which may be accomplished by using yeasts and other microorganisms (reviewed by Gadd, 2000). For example, *S. cerevisiae* is very effective in sequestering zinc, and the potential exists to use yeast, including residual yeast from fermentation industries, to biosorb zinc from effluents (e.g., from the electroplating industry). It may be possible to recover/recycle zinc from yeast. The cell wall plays an important role in zinc sequestration by yeast (White and Gadd, 1987). For example, Hall (2001) has shown that in actively dividing, viable cells of *S. cerevisiae*, most zinc is soluble (vacuolar), while in starved or nonviable cells, most zinc is insoluble (cell wall). This means that dead yeast cells, or even yeast cell wall preparations, could potentially be used in bioremediation of zinc from industrial process effluents.

B. Biomineral Nutrition

This relates to the use of yeast in human dietary supplements as sources of trace minerals. *S. cerevisiae* possesses several attributes as a biomineral nutrient including its safety/non-pathogenicity, availability/economy, well-developed technology, public acceptability, and nutritional value. Regarding the latter, yeast cells comprise the following cellular constituents: proteins (~50%), carbohydrates (~30%), lipids (~5%), nucleic acids (~10% RNA), vitamins, antioxidants, and

minerals. Easily grown and readily available yeasts such as baker's or brewer's strains of *S. cerevisiae* represent excellent natural sources of essential metals such as K, Mg, Ca, Fe, Mn, and Zn, and this yeast can be further artificially enriched with several other inorganic micronutrients including selenium, molybdenum, and chromium. Such yeasts are now commercially produced as effective carriers of these trace elements for use in alleviation of dietary deficiencies in humans and animals.

C. Beverages

The ability of yeast cells to accumulate metals may be usefully exploited in alcoholic beverage biotechnology. For example, Smith and Walker (2000) have investigated the potential of using metal-enriched *S. cerevisiae* to improve fermentation performance. They have shown that Mg-preconditioned distiller's or brewer's yeast, with elevated levels of cellular magnesium, were more fermentatively active compared with non-preconditioned cells with normal levels of cell magnesium and also displayed increased tolerance to stress. Mineral-enriched yeasts have potential in addressing the problem of insufficient bioavailable metal ions for optimal fermentation performance by yeast and some commercial products (e.g., zinc-enriched *S. cerevisiae*) are now available as fermentation supplements. Such products may also be acceptable for use in German breweries and Scotch whisky distilleries that do not allow mineral supplements (in the form of inorganic salts) because of national legislative restrictions.

D. Bioethanol

Over 30 billion liters of ethanol are produced per annum, and around 60% of this is for fuel use. Bioethanol—that is, fermentation alcohol destined for fuel use (as both an extender and as an additive to gasoline)—is already produced on a large scale in Brazil and North America and is set to increase significantly in the UK and in Europe. The substrates currently employed are sucrose (juice and molasses), and starch (cereals), but there is potential in exploiting lactose (from cheese whey), fructose (from plant tuber inulin), and cellulose/lignocellulose (from forestry and agriculture) in the future. The ''ideal'' yeast for bioethanol production would possess the following characteristics: rapid and efficient fermentation (with minimal yeast growth and foaming characteristics), consistently low production of secondary fermentation metabolites (glycerol, fusel oils), stress tolerance (ethanol,

osmotic, temperature, acid, bacteria), appropriate flocculation characteristics, high viability and vitality for recycling/pitching and genetic stability. It may be possible by using metal-enriched yeast seed cultures to improve some yeast physiological characteristics (such as fermentation efficiency and stress-tolerance), which would benefit bioethanol producers. Walker and Smith (2000) have already shown that magnesium preconditioned *S. cerevisiae* exhibit improved fermentation performance and increased stress-resistance. Smith (2001) further showed that elevated cellular magnesium content of preconditioned yeast correlated with increased activity of pyruvate decarboxylase, a key enzyme of fermentative metabolism. On a similar vein, Hall (2001) showed a correlation between cell zinc content and alcohol dehydrogenase activity in industrial strains of *S. cerevisiae*. Such physiological *cell engineering* of yeasts holds promise for the fermentation industries at a time when there is reluctance to embrace genetic engineering (at least for food and potable alcohol producers). It is clear, however, that further exploitation of metal-enriched yeast for alcohol fermentation processes requires more research in terms of metal uptake, cellular localization, and utilization.

VI. Metals and Yeast Fermentation Processes

A. Metals Important in Fermentation

The mineral nutrition of yeasts is relevant to brewers, winemakers, distillers, and bioethanol producers as they seek to increase fermentative capacity, improve ethanol yields, and maintain product consistency. The nature and concentration of metal ions in fermentation media are indeed important factors that influence yeast cell physiology and production of yeast fermentation commodities. The most important metals that influence yeast fermentation processes are potassium and magnesium (as bulk metals) and calcium, manganese, iron, copper, and zinc (as trace metals). Stewart and Russell (1998) and Boulton and Quain (2001) have discussed the roles of bulk and trace metals in relation to brewing yeast fermentation processes. In relation to brewing, most interest to date has focused on the roles of zinc and calcium in influencing wort attenuation and yeast flocculation, respectively.

Zinc is an essential micronutrient for yeast, and occasionally brewer's wort may be Zn-deficient, resulting in impaired fermentation performance (Bromberg *et al.*, 1997; Densky *et al.*, 1966; Desmartez, 1993; Rees and Stewart, 1998; Stehlik-Thomas *et al.*, 1997). This phenomenon, which can lead to slow, or so-called ''sluggish,'' fermentations in

breweries, is yeast strain-dependent but may be encountered when wort zinc levels are below around 0.1 ppm. Zinc plays a major role in yeast fermentative metabolism because it is essential for ethanol dehydrogenase activity (the terminal Zn-metalloenzyme in alcoholic fermentation—see Magonet *et al.*, 1992), but it can also stimulate uptake of maltose and maltotriose into brewing yeast cells, thereby augmenting fermentation rates. Table VI summarizes important roles for zinc in yeast physiology.

Calcium requirements for yeast fermentation are arguable. Certainly, a clear-cut requirement for external calcium ions for growth of yeast cells (and other microbial cells) has yet to be demonstrated (Youatt, 1993). Cells actively exclude calcium to maintain sub-toxic cytosolic levels and intracellular calcium concentrations are further controlled by specific Ca-binding proteins such as calmodulin. Calcium's role in yeast fermentation processes appears to be mainly as an extracellular cation. For example, calcium may act as a protector of certain secreted proteins (such as hydrolytic enzymes) and as a facilitator of yeast–yeast interaction during flocculation (Miki *et al.*, 1982) and agglomeration (Guinard and Lewis, 1993). The presence of excess calcium in fermentation media (e.g., in molasses, malt wort, etc.) can inhibit yeast growth (Saltokoglu and Slaughter, 1983) and fermentative activity (Walker *et al.*, 1996). These effects of calcium may be expressed at the level of direct inhibition, or through antagonism with other essential cations, notably magnesium. Calcium can detrimentally affect yeast physiological functions by antagonizing magnesium uptake and by suppressing

TABLE VI

ROLES FOR ZINC IN YEAST PHYSIOLOGY PERTINENT TO FERMENTATION PROCESSES

Role	Examples
Enzyme activity	Dehydrogenases (e.g., alcohol dehydrogenase, glutamate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, aldehyde dehydrogenase), cysteine desulphydrase, carbonic anhydrase, carboxypeptidase A & B, alkaline phosphatase, α-mannosidase, aldolase, superoxide dismutase, DNA/RNA polymerase, ribonuclease
Protein structure maintenance	Zn-finger DNA binding proteins
Cell surface integrity	Promotes yeast flocculation, stabilizes cell membranes
Sugar uptake	Stimulation of maltose and maltotriose uptake
Miscellaneous	Activation of riboflavin synthesis

magnesium-dependent enzymes. Calcium-magnesium antagonism with respect to yeast fermentation processes is discussed further below (Section VI.C). Other trace metals that may influence fermentation include manganese, copper, and iron. These are required for yeast metabolism as enzyme cofactors (especially Mn) and in yeast respiratory pathways as components of redox pigments (especially Fe and Cu).

Until relatively recently, little attention has be paid to the roles of magnesium in yeast physiology and fermentation performance. Magnesium ions participate in myriad physiological processes in yeast cells including cell division cycle progression, intermediary and biosynthetic metabolism, environmental stress-protection (Table VIII), and the general maintenance of cell viability and vitality (see Table VII). For industrial yeasts such as *S. cerevisiae*, magnesium is absolutely essential for growth and metabolism, and the bioavailability of this cation in media such as malt wort (Walker *et al.*, 1996), molasses (Chandrasena *et al.*, 1997), and wine must (Birch *et al.*, 2003) is now recognized as being very important for efficient industrial fermentations with this yeast.

In alcohol fermentations, magnesium ions can directly influence the rate of yeast growth, sugar consumption, and ethanol production (Rees and Stewart, 1999; Saltokoglu and Slaughter, 1983; Walker *et al.*, 1996).

However, important questions remain regarding magnesium and other metals in industrial fermentation processes. For example: Do growth media metal ion levels remain constant? Is there sufficient *bioavailable* metal ion for optimal enzyme action/fermentation? Do levels

TABLE VII

ROLES FOR MAGNESIUM IN YEAST PHYSIOLOGY PERTINENT TO FERMENTATION PROCESSES

Role	Examples
Enzyme action	Essential cofactor for numerous (over 300) enzymes, especially those required for glycolysis (including pyruvate decarboxylase)
Cell viability and growth	Magnesium absolutely required for cell division cycle progress in yeast (stimulates DNA synthesis and onset of mitosis). Yeasts have high growth demands for magnesium (low Ks values—see text). Cells can be synchronised into division using a Mg starve-feed regime
Cell and organelle structure	Membrane stabilization, ribosome and mitochondria structure
Stress-protectant	Counteracts stresses caused by temperature, osmotic pressure, oxygen free radicals, heavy metals (see Table VIII)

TABLE VIII

ANTI-STRESS FUNCTIONS OF MAGNESIUM

Stress	Comments
High and low temperatures	Magnesium maintains cell viability when cells are heat or cold shocked. Magnesium prevents synthesis of heat-shock proteins
Oxidative stress	Magnesium counteracts stress caused by reactive oxygen species. Magnesium deficit contributes to cellular ageing linked to free radical cellular damage. Mg-deficient cells are more susceptible to *in vivo* oxidative stress causing lipid peroxidation (magnesium causes a significant fall in malonyl dialdehyde and increase in reduced glutathione) by neutralizing O_2 free radicals
Ethanol toxicity	Ethanol increases yeast cell permeability to magnesium. Magnesium increases tolerance to otherwise toxic levels of ethanol
Heavy metals	Magnesium counteracts the toxic effects of Cd, Co, Cu, Al

of certain metals antagonise beneficial effects of others? The following sections attempt to provide some insight into these questions.

B. BIOAVAILABILITY OF METALS IN INDUSTRIAL MEDIA

The major factors that affect yeast fermentation performance, particularly for the production of ethanol, are yeast strain (genotype), nutrients, physical conditions, and competitive microbes (notably wild yeasts and bacteria). Mineral nutrients should be given careful attention, because efficient conversion of carbon source (e.g., sugar) to desired product (e.g., ethanol) by fermentation depends not solely on the available fermentable carbon but also on the bioavailability of essential metal ions. Metal composition of fermentation media will vary greatly depending on raw materials and process conditions. Therefore, any factor that reduces metal bioavailability and compromises metal ion uptake will in turn adversely affect yeast growth and fermentative activity. An important question that arises is, are the minerals supplied in industrial fermentation media bioavailable for yeast cell assimilation? Bioavailability depends on metal solubility and the properties of metal-complexing ligands. Generally, industrial fermentation feedstocks such as molasses and malt wort contain many metal chelating and absorbing components that can reduce bioavailability. The levels of un-complexed, un-absorbed, and un-sequestered metals in yeast growth media represent biologically free levels and are much more meaningful than total levels (as discussed by Hughes and Poole, 1991). Free metals represent bioavaiable metals and

attention to metal bioavailabitiy may prevent slow and premature fermentations conducted by yeast. For magnesium ions in fermentation, the following considerations are important:

1. Yeast demand for Mg during fermentation is high (for glycolytic enzyme activity)
2. Free (biologically available) Mg may not be sufficient to meet this demand
3. Ca antagonism reduces Mg uptake and Mg bioavailability
4. Increasing free Mg in stimulates fermentation.

By increasing magnesium ion bioavailability, either extracellularly with media supplements (Walker *et al.*, 1996) or intracellularly by yeast cell preconditioning (Smith and Walker, 2000; Walker and Smith, 1999), certain improvements become evident in yeast fermentation performance and in cellular stress protection. In industrial fermentations, magnesium bioavailability may be augmented by supplementing media with magnesium salts (e.g., magnesium sulfate), by using magnesium-enriched (or preconditioned) yeast or by using proprietary yeast ''foods.'' The latter have multifunctional roles such as alleviation of CO_2 inhibitory effects and provision of extra sources of assimilable nitrogen (hydrolysed protein), vitamins, and metal ions (to increase bioavailability). Magnesium supplements have been shown to improve fermentation in the following industrial feedstocks: molasses, malt wort, cheese whey, wine must (Walker *et al.*, 1996).

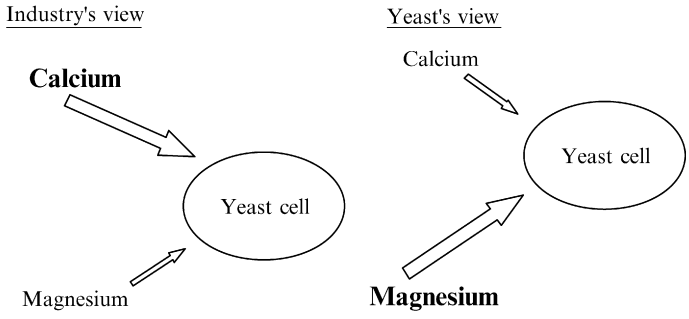
In summary, several factors may reduce the bioavailability of essential metal ions in yeast fermentation processes, including the makeup of the media employed, processing conditions, and the presence of antagonistic and toxic metals. However, several relatively straightforward strategies can be adopted to counteract such reduction.

C. Metal–Metal Interactions

Interactions between metals in fermentation media can influence essential metal ion bioavailability and, consequently, yeast physiology. An imbalance of mineral nutrition, particularly with respect to metal–metal antagonism, can result in complex alterations in yeast growth and metabolism. Metals may compete with each other for binding sites on and in yeast cells, and they may act antagonistically toward each other in terms of biochemical functions. Knowledge of metal–metal interactions is important in media optimization studies. Chandrasena *et al.* (1997) have investigated metal ion interactions in yeast fermentations, particularly with regard to K, Mg, Ca, and Zn interactive effects on

alcohol production by *S. cerevisiae*. Fermentation media were designed to simulate high, intermediate, and low levels of K, Mg, and Ca in molasses and similarly for Mg, Ca, and Zn in malt wort. Subsequent ANOVA (analysis of variance) of fermentations with these levels of metals showed that alcohol production by yeast depended on complex interactions among the relevant metals. It was found that for a fixed level of Mg in molasses, ethanol production varied with changing levels of Ca and K in a predictable way (a response surface model fitted). In addition, for high levels of Mg, the model showed that certain combinations of K and Ca could maximize ethanol production following molasses fermentations. In malt wort, Mg, Ca, and Zn were found to exert significant interactive effects on fermentation, and it was concluded that statistical modeling with response surfaces had the potential to predict fermentation performance in media with variable levels of metal ions.

In terms of antagonistic interactions, the biochemical antagonism between magnesium and calcium may have practical implications for yeast fermentation industries. Many enzymes, particularly several transphosphorylases of glycolysis, have specific and essential requirements for magnesium, and these enzymes are inhibited by calcium ions that bind competitively to them (Heaton, 1990; Kaim and Schwederski, 1994; Walker, 1999b). Magnesium is absolutely required as a cofactor for numerous enzymes in cells, but relatively few enzymes by comparison need calcium. Other physiological differences between magnesium and calcium include the active cellular inclusion of magnesium, but the active exclusion of calcium. This is reflected in major cellular concentration differences between the two cations; intracellular free magnesium is around 0.5–1.0 mM, while calcium is maintained at submicromolar levels (around 100 nM). Unfortunately, this differential cellular demand for magnesium and calcium is not met by industrial yeast growth media, many of which contain calcium levels that are



much higher than magnesium (Walker, 1994). This physiologically anomalous situation can be signified by the following concept:

For example, cellular Mg:Ca ratios may be as high as 1000:1 (for intracellular free ions), but media Mg:Ca ratios may be as low as 0.1:1 (for some types of molasses). In other words, some industrial fermentation media may not be satisfying yeast physiological requirements for these particular metals. Walker (1999b) has discussed the biotechnological significance of magnesium-calcium antagonism, which in yeast fermentation processes is manifest by calcium counteraction of magnesium stimulatory effects. Basically, by increasing Mg:Ca ratios in fermentation media, Walker *et al.* (1996) found it possible to improve alcohol production. This was presumably caused by suppression of the inhibitory effects of calcium on magnesium uptake and cellular utilization. Careful adjustments of external magnesium and calcium concentrations are therefore viewed as a relatively simple means of manipulating yeast fermentation performance.

D. Demand for Metals by Yeast During Growth and Fermentation

During fermentation, yeast cells take up metals to satisfy various physiological needs. Such needs are nutrient uptake, growth, cell division, energy transduction, and survival in the face of stress. Cellular uptake and subsequent metabolic utilization of metal ions are prerequisites for maximizing fermentation performance by yeast. This is especially evident for metals that are essential cofactors for glycolytic and alcohologenic enzymes. Magnesium and zinc are two such metals.

For magnesium, Walker and Maynard (1997) have shown that a close relationship exists between fermentative activity of *S. cerevisiae* and magnesium accumulation from growth media. Cellular demands for magnesium during fermentation were reflected at different stages of fermentation, such that entry of cells into stationary phase (coinciding with the time of maximum ethanol and minimal sugar concentrations) correlated with periods of maximal magnesium uptake. Lentini *et al.* (1990) have shown similar patterns in brewing fermentations. Walker and Maynard (1997) further proposed that magnesium taken up, and subsequently released by yeast during fermentation represented cytosolic free magnesium required as a metabolic cofactor.

For zinc, Hall (2001) and De Nicola and Walker (unpublished observations) have shown that fermenting cells of *S. cerevisiae* take up this metal very rapidly from their growth medium. Figure 2 shows a typical pattern of zinc uptake observed by industrial strains of this yeast. From these data, it appears that yeast demand for zinc is immediate during

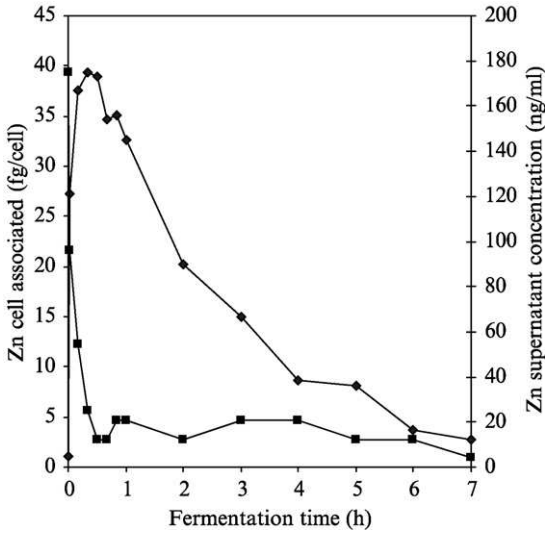


FIG. 2. Zinc uptake by a brewing strain of *S. cerevisiae*. Cells of an industrial ale yeast were inoculated into malt wort (original gravity, OG 1060) at 28 °C. Zinc was analyzed during the initial stages of fermentation (first 7 hours) in both cells (◆) and supernatant (■) by using atomic absorption spectrophotometry.

the initial stages of fermentation. It is most likely that a large proportion of this zinc is simply cell wall-bound (biosorbed), and Hall (2001) has provided evidence of such an interaction during fermentation.

It is important to remember that *S. cerevisiae* is able to perform fermentation or respiration, and this yeast has therefore been described as a facultative organism in terms of sugar catabolism. Fermentative or respiratory modes of metabolism in this yeast will predominate depending on the availability of oxygen and glucose. Two regulatory phenomena describe how *S. cerevisiae* responds to alterations in oxygen and glucose availability: The Pasteur Effect and The Crabtree Effect. Basically, the former states that fermentation is faster in the absence of O_2 (i.e., cells respond to energetic discrepancies (lack of ATP) by increasing the rate of glucose catabolism under anaerobic conditions), and the latter states that fermentation predominates, even in the presence of O_2, because high sugar levels suppress respiration. This means that in industrial fermentations when sugar levels are high (generally when they exceed 0.1% w/v for *S. cerevisiae*), the Crabtree effect is much more relevant than the Pasteur effect. Several possible reasons for the Crabtree effect have been proposed: catabolite repression (Gancedo, 1992), catabolite inactivation (Wills, 1990), limited

respiratory capacity (Käppeli and Sonnleitner, 1986), and magnesium availability (Walker, 1994). Considering the latter, it has been shown that magnesium ions dramatically affect mitochondrial structure and control switches from respiration to fermentation in the Crabtree-positive yeasts. Magnesium may therefore influence expression of Crabtree effect, and Walker (1994) has hypothesized that intracellular magnesium may control metabolic flux at level of pyruvate. In essence, this hypothesis proposes that pyruvate decarboxylase (which channels carbon down the fermentative pathway) and pyruvate dehydrogenase (which channels carbon down the respiratory pathway) possess low and high affinities for intracellular free magnesium ions, respectively. Smith (2001) has provided some support for such a hypothesis by demonstrating a close relationship between intracellular magnesium and pyruvate decarboxylase activity in brewing strains of *S. cerevisiae*. Such a model has practical implications for industrial fermentation processes because it may be feasible to promote either respiration (for maximizing yeast biomass) or fermentation (for maximizing ethanol) solely on the basis of manipulating magnesium bioavailability.

E. Metals and Yeast Stress During Fermentation

In the fermentation industries, the viability and vitality of the culture yeasts are crucially important for ensuring process efficiency and product quality. Unfortunately, yeasts used for industrial fermentation processes may be subject to a variety of chemical, physical, and biological stresses that affect adversely yeast growth and metabolic activity (reviewed by Walker, 1998a). The major stresses encountered by yeast are summarized in Fig. 3, and for *S. cerevisiae* alcohol fermentations, the principal stress factors are temperature shock, osmostress, and ethanol toxicity.

An understanding of stress physiology in yeast cells is necessary to counteract the deleterious effects of stress on fermentation performance. Depending on the particular stress, yeast cells evoke stress responses in an effort to ensure survival when they are exposed to environmental insults, and these include the following:

1. Increased synthesis of trehalose and glycerol
2. Induction of heat/cold shock protein biosynthesis
3. Stress enzyme induction (e.g., ATPase, superoxide dismutase)
4. Cell membrane structural changes
5. Production of glutathione
6. Modulation of ionic homeostasis.

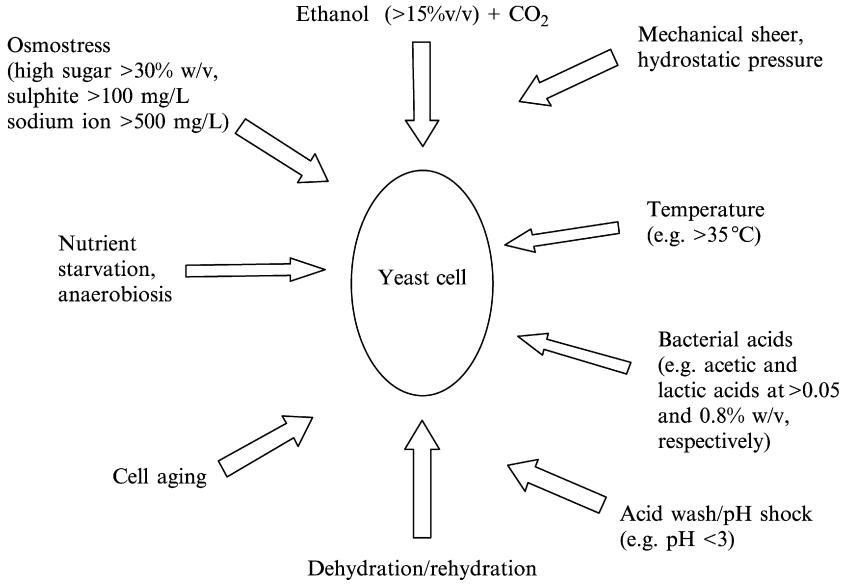


FIG. 3. Stress factors in yeast cells used in fermentation.

Concerning the latter, both heat shock and ethanol can lead to disruption of cellular ionic homeostasis, and this can lead to yeast cell death. Walker (1998b) has shown that these stresses induce significant leakage of magnesium ions from brewing strains of *S. cerevisiae*, and such leakage correlated to loss of culture viability. It has further been shown that increasing magnesium ion availability, either through external media supplementations or through cellular magnesium enrichment (or preconditioning), resulted in physiological protection being conferred on cells exposed to otherwise lethal heat shock or toxic ethanol (Walker, 1998b). In a study with wine yeasts, Birch and Walker (2000) showed that cultures propagated in elevated levels of magnesium (20 mM, as opposed to 2 mM) led to repression of heat shock protein biosynthesis following thermostress or ethanol toxicity. Several yeast studies have now implicated magnesium as a cellular protectant against osmostress (D'Amore *et al.*, 1988), ethanol (Birch and Walker, 2000; Ciesarova *et al.*, 1996; Dombek and Ingram, 1986; Walker, 1998b), and toxic metals like manganese (Blackwell *et al.*, 1997), copper (Karamushka and Gadd, 1994), cadmium (Kessels *et al.*, 1985), aluminium (MacDiarmid and Gardner, 1996), and cobalt

(Aoyama *et al.*, 1986). There is also evidence from animal cells that magnesium can act as an antioxidant by neutralizing the effects of oxygen free radicals and by increasing levels of intracellular glutathione (Durlach, 1988; Rayssiguier *et al.*, 1993; Szantay, 1995). Anti-stress functions of magnesium are summarized in Table VIII.

Magnesium may be exerting a general stress-protective role in yeast cells by charge-neutralization of membrane phospholipids, resulting in a stabilization of the lipid bilayer and a decrease in membrane fluidity (Walker, 1999b).

The practical implications of this for yeast fermentation industries is that magnesium-replete cultures are much more likely to withstand the rigors of industrial processes than are magnesium-limited cultures.

VII. Conclusions and Future Prospects

This review has highlighted the important roles of metals in yeast fermentation processes. In yeast cell physiology, these roles are multifarious and can affect significantly the progress and efficiency of industrial fermentations. For *S. cerevisiae* cell physiology, the following are some of the salient points that have been raised herein: metal ion *bioavailability* in fermentation media is more important than total levels of metals; high calcium levels are detrimental; metal-preconditioned yeasts may improve fermentative metabolism; stress affects metal ion (e.g., Mg) homeostasis and some metals can counteract physiological stress.

There are several industrial implications arising from the research discussed in this chapter. First, it is evident that many metals strongly influence yeast fermentation performance, and more careful attention should be paid to minerals in fermentation feedstocks than has hitherto been the case. This author is of the opinion that metals are as equally important as carbon and nitrogen sources in industrial media used for optimisation of yeast fermentation processes. Second, by physiologically adapting starter yeast cultures, for example using metal-preconditioning, benefits may accrue in terms of improved fermentations. For brewers, winemakers, and distillers, such an approach may circumvent any reluctance, or necessity, to supplement fermentation media with additional mineral salts. Third, industrial yeast fermenters represent stressful environments for yeast cells. However, certain metals may minimize such stress, particularly that caused by extremes in temperature and ethanol concentrations, by conferring a degree of cell membrane protection. Magnesium is the prime candidate for a yeast stress-protectant in fermentation processes.

This review has focussed on *S. cerevisiae* and traditional fermentations such as alcohol production. Nowadays, many non-*Saccharomyces* yeasts are employed in bioreactors for production of high-value pharmaceutical commodities. While we are gradually accumulating useful fundamental information on the mineral nutrition and metabolism of *S. cerevisiae*, which may prove of practical value, unfortunately, we have only scratched the surface of similar knowledge for the massed ranks of non-conventional yeasts. Only when we understand how metals interact with these organisms will biotechnologists be able to fully exploit yeast biodiversity.

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Interactions between Lactobacilli and Antibiotic-Associated Diarrhea

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I. Introduction

The host and its indigenous (normal) microflora together form a well-functioning ecological system. Various exogenous and endogenous influences direct the balance/imbalance of the system. Antibiotics are the most common and effective drugs used for treatment of infectious diseases. However, there is increased awareness of the fact that the use of antibiotics often disturbs the indigenous protective microflora. Even sub-inhibitory concentrations of antibiotics lead to imbalance of the normal microflora and create a more pathogenic biofilm covering mucosal surfaces (Cuperus *et al.*, 1995). Recovery of the indigenous flora may take weeks to months (Larson and Borriello, 1990). During this time the person is predisposed to diseases from pathogenic or opportunistic bacteria.

Antibiotic-associated diarrhea (AAD) is probably the most common manifestation of normal microflora alteration caused by antimicrobial treatment, with an incidence of up to 30 per 100 hospitalized patients

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(McFarland, 1998). AAD may be divided into two types: uncomplicated or nonspecific AAD, and *Clostridium difficile*–associated diarrheas (CDAD). To the first group usually belong mild and self-limited diseases, yet CDAD could be complicated with colitis, and it causes a major burden on the health care system (Wilkins and Lyerly, 2003). Since the main cause of AAD is alteration of intestinal microflora, the prophylaxis and therapy for restoring normal microflora seems to be the most natural approach with these groups of diseases. Unfortunately, it is hard to assess the specific contributions of different microorganisms of indigenous microflora in a well-balanced system with the host.

In this chapter we summarize the studies concerning the impact of different external factors, particularly antibiotics, on various groups of indigenous microflora and evaluate experimental animal models of CDAD. This approach could predict which groups of microbes are able to restore the normal symbiosis and help to develop further applications of microbial interference therapy and prophylaxis against *Clostridium difficile* infections. The chapter also summarizes some human clinical trials with non-pathogenic commensal lactobacilli that have been considered as probiotics with beneficial health effects, including prevention or amelioration of AAD.

II. Microbial Ecology of Intestinal Tract and Colonization Resistance

A. Indigenous Microflora

The gastrointestinal tract (GI) of a human is colonized with more than 400 different species of microorganisms in numbers of more than 10^{11}–10^{12} per gram (reviews of Macfarlane and Cummings, 1999; Simon and Gorbach, 1984). The term *indigenous microflora* (IMF) signifies the groups of non-pathogenic or potentially pathogenic microbes that are permanently inhabiting a particular biotope (lumen or mucosa; ileum, jejunum, or colon) of a particular individual and are in symbiotic association with the host (Rusch, 1989; Savage, 1987).

There are great individual differences in the quantitative and qualitative composition of intestinal microflora, although the stability of microflora in particular individuals has been demonstrated (Meijer-Severs and Santen, 1986; Mikelsaar, 1992). Recently, with molecular methods (i.e., denaturing gradient gel electrophoresis [DGGE]), it was proved that the predominant mucosa-associated bacterial community along the colon was host specific and significantly different from the fecal community (Zoetendal *et al.*, 2002). The individually different quantitative composition of fecal microflora depends on

the host genetics, as shown by investigating adult monozygotic twins (Mikelsaar *et al.*, 1984). Monozygotic twins reveal the identity of many genetic markers (i.e., antigenic structure of somatic cells and secretions of the host, as well as the immune reaction, that are important for the selective colonization by the indigenous microflora (Warner *et al.*, 1988). To date, the same relationship has been confirmed by using molecular methods by comparing the intestinal microflora of genetically identical monozygotic twins (Zoetendal *et al.*, 1998).

In the large intestine, relying on cultivation assays, the predominant anaerobic bacteria are Gram-negative rods such as *Bacteroides* and *Fusobacterium* sp., Gram-positive rods such as *Bifidobacterium* sp., *Eubacterium* sp., *Clostridium* sp. and cocci such as *Peptostreptococcus* sp., *Veillonella* sp. (Hentges, 1983; Levy, 2000; Mikelsaar and Mändar 1993; Sepp *et al.*, 1997).

More anaerobes can be detected by 16S rDNA probes than by cultivation techniques (Harmsen *et al.*, 2000; Štšepetova *et al.*, 2002). The molecular techniques have identified new groups of bacteria (*Ruminococcus* sp., *Phascolarctobacteria* etc.) colonizing the intestinal tract in high numbers. However, the anaerobes like bacteroids, clostridia, and eubacteria (accounting for 20% to 29%), and bifidobacteria (accounting for 3% of the total fecal population) are still among the most important predominant microbes (Franks *et al.*, 1998). In the future, the applicability of new techniques (genomics, proteomics, metabolomics) can profoundly enhance our knowledge about the various components of the indigenous microflora.

Apart from indigenous microflora, each biotope consists of non-indigenous (transient, allochtonous) microbes originating from the environment or from IMF of the other biotopes (Rusch, 1989). These pathogenic or opportunistic pathogens (e.g., *C. difficile*) can inhabit a biotope either for a short time or in the case of more profound perturbation of the microbial ecosystem, even for prolonged periods.

B. Lactic Acid Bacteria as Part of Normal Microflora

Lactobacilli are the the well-known component of intestinal microflora. Lactobacilli are Gram-positive, rod-shaped, facultatively anaerobic, non-sporulating, acid-tolerant, and catalase-negative bacteria with a DNA base composition of less than 53 mol% G + C. Lactobacilli can be divided into subgenera such as *Thermobacterium, Streptobacterium, and Betabacterium* according to their growth temperatures and hexose fermentation pathways (Kandler and Weiss, 1986). Modern molecular methods based on the comparison of highly conserved molecules of

16S ribosomal ribonucleic acid (16S rRNA) genes have shown that these subgroups are inconsistent with the phylogenetic relationship of the species within the genus (Song *et al.*, 2000). The principal phylogenetic grouping of *Lactobacillus* spp. proposed is summarized as follows: (1) *L. delbrueckii* group; (2) the *L. casei–Pediococcus* group; and (3) the *Leuconostoc* group including the species from the genera *Lactobacillus, Oenococcus*, and *Weissella* (Stiles and Holzapfel, 1997).

In addition, based on the peptidoglycan type of the cell wall and their fermentation pathways for pentoses and hexoses, lactobacilli are divided into obligately homofermentative lactobacilli (OHOL), facultatively heterofermentative lactobacilli (FHEL), and obligately heterofermentative lactobacilli (OHEL) (Hammes and Vogel, 1995). The latter division is valuable to understand their physiology and impact on human health.

There are few studies on the prevalence of lactobacilli in the small intestine. Usually, the numbers of microorganisms in the proximal jejunum are approximately 10^4/ml, while the oropharyngeal microflora predominates. However, in a recent study *Lactobacillus* sp. microorganisms were found only in two healthy subjects out of 20 (Sullivan *et al.*, 2003). In contrast, in the fecal samples, the *Lactobacillus* strains were present in approximately 70% of adults who consume a Western-like diet (reviewed by Heilig *et al.*, 2002). In elderly persons the prevalence of lactobacilli was even higher, reaching 90% (Mikelsaar *et al.*, 1998; Speck, 1976).

Earlier studies have demonstrated that the *Lactobacillus* spp. counts reached 10^{10} CFU/g in the fecal microflora of adults and were outnumbered only by obligate anaerobes (Simon and Gorbach, 1984). More recent investigations have shown counts of $10^{8–9}$ CFU/g (Sepp *et al.*, 1997). A good marker for characterisation of the intestinal microflora is the distribution of particular groups of microorganisms in the total count. The relative abundance of lactobacilli in the total count of fecal bacteria for particular persons is $<2\%$ in children up to 1y and $<0.1\%$ in adults (Mikelsaar and Mändar, 1993; Sepp *et al.*, 1997). These data have been confirmed by Sghir *et al.* (2000) showing that lactobacilli constitute less than 1% of the total bacterial community within human fecal microbiota.

Using a fluorescent *in situ* molecular hybridisation technique (FISH), Marteau *et al.* (2001) found that the counts of cecal lactobacilli were quite similar to those of fecal lactobacilli (8.4 vs. 8.8, log CFU/g, respectively). Converserly, their distribution in cecal and fecal samples was quite different (23% vs. 7%). This finding may stress the importance of lactic acid producing bacteria in mucosal flora of the caecum,

the particular biotope most frequently attacked by pathogenic bacteria like *C. difficile*.

The lactoflora of the human GI tract consists of various species, subspecies, and biotypes of homo- and heterofermentative lactic acid bacteria. The most frequently occurring lactobacilli belong to 6 species: *Lactobacillus acidophilus-group, L. salivarius, L. casei, L. plantarum, L. fermentum, L. brevis* in various combinations (Mikelsaar *et al.*, 1998; 2002; Molin *et al.*, 1993; Reuter, 1997; Song *et al.*, 1999). The *L. acidophilus* group has now been divided into *L. acidophilus sensu strictu, L. gasseri, L. crispatus* and *L. johnsonii* (Holzapfel *et al.*, 2001). In addition to these, the frequent occurrence of *L. reuteri* in the GI tract of humans and animals has also been shown (Axelsson, 1990; Kandler and Weiss, 1986; Reuter, 1997).

Rectal biopsies from 42 individuals showed that lactobacilli counts ranged from <2.0 to 7.0 log CFU/g mucosa, with a median of 4.0 log CFU/g (Ahrné *et al.*, 1998), while the most frequently isolated species from rectal mucosa were *L. plantarum* and *L. rhamnosus*. This finding hints on some geographical differences, as in studies of Russian astronauts (Lencner *et al.*, 1984) and Italian elderly people *L. rhamnosus* was very seldom found (up to 20%) in individuals (Silvi *et al.*, 2003). Additionally, the geographic differences were apparent when comparing the fecal *Lactobacillus* species composition of Estonian and Swedish 1- to 2-year-old children (Mikelsaar *et al.*, 2002).

Large individual differences complicate the picture of GI lactoflora even more. In a survey over a 15-year period of 10 healthy volunteers, the stable persistence of fecal *Lactobacillus* species were revealed for each person even though the persons aged during the study, thus having several health failures, and used some medicines (Mikelsaar and Mändar, 1993; Mikelsaar *et al.*, 1998). Similarly, Kimura *et al.* (1997) showed by pulsed-field gel electrophoresis (PFGE) that eight of ten subjects tested harbored a unique collection of lactobacilli in the intestine.

C. Colonization Resistance

The term microbial colonization resistance (CR) has been defined as the limiting action of the IMF on colonization of the bowel by exogenous as well as endogenous potentially pathogenic microorganisms. According to van der Waaij and Berghuis (1974), the CR relies on the anaerobic microflora of the gut. Several studies indicate that anaerobic IMF really has the main role in microbial CR, and aerobic potentially pathogenic microorganisms do not contribute (Borriello,

1989; Vollaard and Clasener, 1994). The set of anaerobes involved in CR has been shown to be quite wide, including some 7 to 10 different species (Boureau *et al.*, 1989). To date, some authors have postulated on the particular role of intestinal lactobacilli in the maintenance of CR (Lidbeck and Nord, 1993; Mikelsaar and Mändar 1993; Naaber, 1997; Salminen and Deighton, 1992). The above-mentioned large geographic and individual variations in the normal microflora of people may obscure the understanding of CR.

The dynamic mechanisms of CR are well reviewed by McFarland (2000), though a full understanding of the complex indigenous microbiota by which protection is offered has not been fully established. Several actions of indigenous bacteria against pathogens, defined as a barrier effect (Borriello, 1990), are possible: competition for nutrients, secretion of antimicrobial substances (bacteriocines, hydrogen peroxide, nitric oxid, short chain fatty acids, proteases etc.), blockage of adhesive receptor sites for bacterial cell wall components or secreted toxins, co-aggregation of bacteria to be removed as larger particles, attenuation of virulence by suppression of toxin production and immune stimulation.

Although microbes play the most important role in maintenance of CR, it could also be mediated by anatomical and physiological factors including salivation, swallowing, and normal gastrointestinal motility; production of gastric acid, lysozyme and mucus protecting the epithelial cells; intact mucosal lining; epithelial cell turnover; secretory IgA levels; and action of M cells, phagocytes, and lymphatic tissue (McFarland, 2000; Rolfe, 1997). Newly described elements of the innate immune system (e.g., antimicrobial peptides such as defensins formed by polymorphonuclear cells and enterocytes) are directed against bacteria (Mahida *et al.*, 1997). It appears, however, that anatomical and physiological CR guaranteeing systems are not capable of keeping the concentration of potentially pathogenic microorganisms under control if the IMF is absent or disturbed.

III. Antibiotic-Associated Diarrhea (AAD)

A. AAD and *Clostridium difficile*-Associated Diarrhea (CDAD)

Gastrointestinal symptoms, particularly diarrhea, are relatively common side effects of antibiotic usage. Antibiotic associated diarrhea designates the diarrheas manifesting during or after recent antimicrobial therapy. AAD not caused by *C. difficile* is usually clinically mild watery diarrhea without complications, and it is resolved after drug

withdrawal. In contrast, CDAD can cause constitutional symptoms (fever and leukocytosis) and severe complications (colitis, bloody diarrhea, toxic megacolon). Symptoms of CDAD often persist after drug withdrawal and relapses are common (Bartlett, 1992).

The reported incidence of AAD ranges from 0.44 to 26/100, depending on host factors and hospitalization status (McFarland, 1998). Although in the majority of cases the exact mechanisms of the side effects of antibiotic consumption are not well understood, the proposed mechanisms include (1) direct action of antibiotics on intestinal function; (2) inducing predisposition to infection with enteric pathogens; and (3) factors secondary to the disturbance of normal intestinal flora that do not involve infection with a known pathogen (Borriello, 1992; Högenauer *et al.*, 1998; McFarland, 1998; Midtvedt, 1989).

C. difficile is the most important causal agent of AAD. From 0.5% to 56% of AAD are caused by *C. difficile,* depending on the patient group investigated. The lowest frequency is observed in non-hospitalized patients. However, probably all nosocomial outbreaks of AAD are caused by *C. difficile* (Bartlett, 1992; De-Barbeyrac *et al.*, 1989; Drapkin, 1992; McFarland, 1998).

1. *Antibiotics*

It is known that nearly all CDADs are related to previous antibiotic treatment and nearly all antimicrobial drugs can induce CDAD. However, several investigations support the idea that the ability of a particular antimicrobial to induce CDAD depends on its spectrum of activity and its pharmacokinetic properties. Antibiotics active against anaerobic bacteria (clindamycin, erythromycin) have been found most frequently associated with CDAD (McFarland, 1993). Administration of third-generation cephalosporins, broad-spectrum penicillins, and clindamycin is associated with the highest risk for CDAD (Bartlett, 1992; Hirschhorn *et al.*, 1994; McFarland, 1993; Stoddart and Wilcox, 2002; Zimmermann, 1991). The challenge with these data is that there exists geographically and individually different compositions of indigenous microflora. The antibiotic causing CDAD in one person may not cause it in the other because of a wide variety of susceptibility to antibacterials of individual predominant microbes of the gut.

Moreover, it seems that the frequency and severity of CDAD do not appear to be antibiotic dose-related, in contrast to AAD that is due to other causes. The assumption is that modification of fecal flora is an essential feature of the drugs, but a confounding interrelated variable is their antibacterial activity against *C. difficile* (Bartlett, 1992). Unexpectedly, according to studies of the hamster model and

of patients, drugs with good activity against *C. difficile* (including ampicillin and vancomycin) may also induce CDAD. Seemingly, it depends on the selective concentration of the drug in the lumen and mucosa of the gut.

B. *Clostridium difficile* and CDAD

1. *Etiopathogenesis*

Clostridium difficile is a Gram-positive spore-forming obligate anaerobe that was first isolated by Hall and O'Toole in 1935 from the feces of an infant and designated as *Bacillus difficilis* (Knoop *et al.*, 1993). Although pseudomembranous colitis (PMC) was described more than a century ago, it started emerging in the 1960s because of increasing use of new antibiotics, particularly after the introduction of clindamycin. The link between PMC and *C. difficile* was made at the end of the 1970s when Larson showed the cytotoxic activity of PMC patients' fecal filtrate on cell culture (Larson *et al.*, 1977). Afterwards, the isolation of *C. difficile* from the intestinal tract of PMC patients and clindamycin treated animals confirmed its etiological role (Bartlett and Gorbach, 1977; Bartlett *et al.*, 1977; George *et al.*, 1978; Larson *et al.*, 1978)

The virulence of *C. difficile* is mainly associated with two toxins, A and B, commonly referred as *enterotoxin* and *cytotoxin*. Until recently, production of toxin A was thought to be the most important factor in the pathogenesis of CDAD. Surprisingly, several outbreaks of toxin A-negative and toxin B-positive strains have been reported during the last decade (Wilkins and Lyerly, 2003). However, in these isolates, toxin B has a broader substrate specificity than both toxins producing isolates. The role of other virulence factors is more obscure. Adhesins have been proposed as being important, but their relevance in the colon is not very clear. The outer cell coat, called the *S-layer,* has also been proposed as an important virulence factor of *C. difficile*. Currently, suppression of indigenous intestinal microflora due to administration of antibiotics, subsequent colonization of the intestinal tract, and production of toxins (A and B or only B) by *C. difficile* appear to be the most important factors for the development of intestinal infection (Bartlett, 1994; Poxton *et al.*, 2001; Wilkins and Lyerly, 2003).

2. *Prevalence*

C. difficile readily colonizes neonates and infants at the time when the microflora succession has not been finished and there is only scarce flora established. In children with mature microbial ecology, the *C. difficile* is cleared from the intestines (Wilson, 1993). Once more the large

geographical differences can be noted as from the Swedish 1- to 2-year-old children with some 34%, while only 4% of Estonians of the same age were colonized with *C. difficile* (Sepp *et al.*, 1997). In healthy adults the carriage rate of *C. difficile* also varies in the intestine of persons from different geographical areas, from 2% in Sweden to 15% colonized in Japan (Knoop *et al.*, 1993) Unfortunately, it is not known if these numbers represent a transient colonization or if *C. difficile* is a permanent component of the stable flora of these subjects (Bartlett, 1994).

Higher age has been reported as a risk factor for colonization by *C. difficile* and CDAD (Bennett and Greenough, 1993). Some 19% of elderly residents have been found colonized by *C. difficile* in long-term-care facilities. Also, the colonization of healthy elderly people in the population is higher than the average for the general population (Nakamura *et al.*, 1981; Simor *et al.*, 1993). The reasons are probably altered CR caused by changes in the gut IMF in older people (Hébuterne, 2003; Hopkins and Macfarlane, 2002). In elderly persons, bifidobacteria decrease or disappear, while lactobacilli, enterococci, enterobacteria, and clostridia increase (Kleessen *et al.*, 1997; Mitsuoka *et al.*, 1990). Hopkins *et al.* (2001), using viable counts and estimations of 16SrRNA abundance, have reported the skewed bifidobacterial results, and a healthy 67-year-old male had, for instance, very high counts of these organisms, belonging to several species. In elderly persons the loss of some properties of the intestinal mucus necessary for adhesion of endogenous bifidobacteria have been assessed (Fang He *et al.*, 2001). Individually different *Lactobacillus* flora was described in the feces of healthy elderly Italian people. However, *L. fermentum* and *B. longum* were the most represented species and suggested the design of functional foods to fortify the intestinal microflora of the elderly (Silvi *et al.*, 2003).

According to different studies, the carriage rate in hospitalized patients varies from 7% to 21%. During an outbreak, even more than 50% of patients in the ward may become colonized by *C. difficile*. Interestingly, from 50% to 85% of these patients may remain asymptomatic, while in others, CDAD develops (Cartmill *et al.*, 1994; Clabots *et al.*, 1992; Johnson and Gerding, 1998; Johnson *et al.*, 1990; McFarland *et al.*, 1990; Simor *et al.*, 1993). It is not fully understood which factors control the population level of *C. difficile* in the gut and the expression of the disease.

3. *CR and* C. difficile

The importance of IMF in maintaining CR to *C. difficile* can most impressively be demonstrated in hamsters: *C. difficile* cannot colonize and cause disease in normal hamsters, but in antibiotic-treated

animals, even small doses of *C. difficile* cause lethal infection, and further, previous administration of fecal microflora of normal hamsters to antibiotic-treated animals prevents CDAD (Borriello, 1990). Despite clear evidence of the protective role of IMF, the exact mechanisms and bacteria that guarantee CR against *C. difficile* remain unknown.

We have found that children colonized with *C. difficile* and patients with CDAD usually have lower counts of lactobacilli in their intestine. Marked individual differences in counts of anaerobes, lactobacilli, and other members of IMF in healthy, as well as in *C. difficile* colonized/infected patients, have been assessed (Naaber *et al*., 1997). Another study has shown quite an opposite trend: in a small number of CDAD patients studied, high prevalence and viable counts of lactobacilli and enterobacteria have been found. However, the decreased total bacterial counts and these of bifidobacteria show simply the influence of previous metronidazole therapy sustaining lactobacilli and coliforms in these four patients (Hopkins and Macfarlane, 2002). It can be speculated that not only the total counts of lactobacilli (or some other bacterial group), but also the species composition of intestinal lactoflora may be important in the maintenance of CR against *C. difficile*.

The antibiotic susceptibility pattern of lactobacilli is not uniform (Table I). Keeping in mind individually different compositions of lactoflora, it can be understood why some people are more prone to CDAD by the same antibiotic prophylaxis than the others. Among antibiotics shown to be associated with the highest risk for CDAD the penicillins, erythromycin, and tetracycline affect most species of human lactobacilli. However, several species of *Lactobacillus* are resistant to cephalosporins of different generations. Cefoxitin and cefuroxime used for prophylaxis in surgery affect the *L. acidophilus* group, *L. brevis* and *L. buchneri* quite differently from the other lactobacilli. Interestingly, the combination of ampicillin and gentamicin often used in control of infections in intensive care seriously affects gastrointestinal lactobacilli. In contrast, treatment of CDAD by metronidazole is most safe for lactobacilli responsible for the maintenance of CR, similarly to vancomycin that suppress only the *L. acidophilus*- group of lactobacilli.

Moreover, the inconsistency of abundant clinical and experimental data indicates that there is no single microbial group that controls the establishment of *C. difficile* in the intestinal tract by a single mechanism. It is more likely that several diverse microbes may be involved by different mechanisms. As IMF varies from person to person, different microbes may have a leading role in CR against *C. difficile* in different individuals.

TABLE I

SUSCEPTIBILITY OF HUMAN *LACTOBACILLUS* SP. (LB) TO MOST COMMON GROUPS OF ANTIMICROBIALS

Antibiotic groups	Species of LB*	Susceptible	Reference
Penicillins	LB	100%	Hamilton *et al.*, 1994
			Felten *et al.*, 1999
			Mändar *et al.*, 2001
Ampicillin	LB	96%	Testore *et al.*, 2002
Methicillin	*L. paracasei*	55%	Testore *et al.*, 2002
Cephalosporins	LB	52–100%	Testore *et al.*, 2002
1st generation			
2nd generation	LB	62%	Charteris *et al.*, 1998
Cefoxitin	*L. acidophilus*-group	19–53%	Mändar *et al.*, 2001
		88%	Testore *et al.*, 2002
	L. brevis	14%	Mändar *et al.*, 2001
	L. buchneri	50%	Mändar *et al.*, 2001
Cefuroxime	LB	43–100%	Mändar *et al.*, 2001
			Testore *et al.*, 2002
	L. brevis	43%	Mändar *et al.*, 2001
3rd generation	LB	50–76%	Testore *et al.*, 2002
Vancomycin	LB	26%	Zarazaga *et al.*, 1999
	L. acidophilus group	93%	Mändar *et al.*, 2001
Macrolides	LB	100%	Muli and Struthers, 1998
Erythromycin			Felten *et al.*, 1999
		62%	Mändar *et al.*, 2001
			Testore *et al.*, 2002
Tetracycline	LB	90–100%	Charteris *et al.*, 1998
			Mändar *et al.*, 2001
			Testore *et al.*, 2002
Aminoglycosides	LB	36–79%	Testore *et al.*, 2002
Gentamycin	microaerobic milieu	93–100%	Mändar *et al.*, 2001
	anaerobic milieu	0%	Charteris *et al.*, 1998
Fluorokinolones	LB	40–100%	Hamilton *et al.*, 1994
			Zarazaga *et al.*, 1999
Ciprofloxacin	*L. acidophilus*,	13%	Mändar *et al.*, 2001
	L. paracasei	94%	Mändar *et al.*, 2001
	L. buchneri	50%	Mändar *et al.*, 2001

(*continued*)

TABLE I (*Continued*)

Antibiotic groups	Species of LB*	Susceptible	Reference
Lincomycin	LB	42%	Testore *et al.*, 2002
Clindamycin	LB	58%	Testore *et al.*, 2002
Metronidazole	LB	0%	Charteris *et al.*, 1998
			Zarazaga *et al.*, 1999
			Mändar *et al.*, 2001

*LB of human origin.

C. Other Causes of AAD

Although *C. difficile* is the most important pathogen that overgrows after antibiotic-induced alteration of intestinal IMF, some other microorganisms are also proposed to cause AAD by this mechanism. These pathogens include *Clostridium perfringens, Staphylococcus aureus, Klebsiella oxytoca,* and *Candida* spp. However, their role, pathogenetic mechanisms involved, and incidence of AAD are not yet fully understood (Borriello, 1992; Högenauer *et al.*, 1998). The overgrowth of a particular species or genus could be an indicator of an imbalance of microflora rather than the real cause of diarrhea.

In most cases of AAD a known specific enteric pathogen cannot be isolated. Since metabolic activity of gut IMF is important for the normal functioning of the intestine, the alteration of intestinal microflora can change the motility of the colon, as well as absorption and secretion. The main mechanisms proposed, carbohydrate malabsorption and decreased metabolism of bile acids, have been described in a recent review by Högenauer *et al.* (1998). These disorders have been mainly associated with a decrease of obligate anaerobes. Some antibiotics such as erythromycin and amoxicillin/clavulanate have been described to affect intestinal motility directly. Since antibiotics always alter intestinal microflora and thus metabolic functions of IMF, it is not clear which mechanisms are predominant in clinical cases of AAD.

IV. *In Vitro* Studies and Animal Experiments

A. Inhibition of *in vitro* Growth of *C. difficile* by Lactobacilli

Several bacterial species isolated from feces have been found to be antagonistic against *C. difficile* on agar plates. These microorganisms include anaerobes (*Clostridium bifermentas, Clostridium beijerinckii,*

Peptostreptococcus productus, Bifidobacterium adolescentis, Bifidobacterium infantis, Bifidobacterium longum) and lactobacilli (*L. acidophilus, L. salivarius*) as well as facultative aerobes such as *Enterococcus* spp., *Streptococcus* spp. (Barclay and Borriello, 1982; Bogovič-Matijašić *et al.*, 1998; Forestier *et al.*, 2001; Lee *et al.*, 2003; Malamou-Ladas and Tabaqchali, 1982; Rolfe *et al.*, 1981; Tvede and Rask-Madsen, 1989). However, in most of these experiments a few IMF strains have been tested against only one indicator *C. difficile* strain.

Reports about *in vitro* activity of lactobacilli against *C. difficile* are controversial. Strus *et al.* (2001) found all tested lactobacilli to be equally antagonistic to *C. difficile* as well as against the other enteric pathogens. Therefore they postulated a similar mechanism of inhibition of lactobacilli against all anaerobic bacteria. Lee *et al.* (2003) found that only 12 strains of 109 lactic acid bacteria tested were antagonistic against one *C. difficile* test strain. Only three of these *C. difficile* suppressing strains were lactobacilli (*L. salivarius*).

To solve this discrepancy, we screened the antagonistic activity of 51 intestinal *Lactobacillus* strains against 23 clinical *C. difficile* isolates and found that five strains (*L. paracasei* and *L. plantarum*) were antagonistic against all and 18 strains were antagonistic against 9 *C. difficile* isolates. Twenty-seven *Lactobacillus* strains had no antagonistic activity against any tested *C. difficile* isolate (Naaber *et al.*, 1998b, 2002). Thus, since the antagonistic activity of lactobacilli as well as the sensitivity of *C. difficile* to this antagonism is strain specific, the results of *in vitro* studies seemingly depend on the selection of indicator strains.

There is little known about the mechanisms involved in this antagonistic activity. Bogovič-Matijašić *et al.* (1998) isolated and characterized two bacteriocins of *L. acidophilus* that showed activity against *C. difficile* strains as well as several other obligatory and facultative anaerobic bacteria. Forestier *et al.* (2001) studied the activity of *L. casei* subsp. *rhamnosus* culture supernatant against *C. difficile* and found that the inhibitory substance was resistant to treatment with protease and heat and had a molecular mass below 3 kDa. They proposed that this could be a bacteriocin-like substance or an organic acid. Several studies support the opinion that acidification of the colonic content with short chain fatty acids produced by lactobacilli and other members of IMF can suppress *C. difficile* growth (Borriello and Barclay, 1986; Ito *et al.*, 1997; May, 1994; Rolfe, 1984; Yamamoto-Osaki *et al.*, 1994). We have also found a close correlation between antagonistic activity of different lactobacilli against *C. difficile* and their H_2O_2 and lactic acid production (Naaber *et al.*, 2002). However, these relationships were not absolute: some highly antagonistic strains were

both H_2O_2 negative and low lactic acid producers. According to these studies several antagonistic compounds of lactobacilli could be involved in the inhibition of *C. difficile*. Whether some of these are important in the maintenance of CR *in vivo* is not completely evident.

B. *In Vitro* Studies of Other Mechanisms Involved in CR

A study carried out by Borriello and Barclay (1986) with a batch culture model showed that the inhibition of *C. difficile* depends on the presence of complete viable intestinal IMF rather than inhibitory substances in culture filtrates. This indicates the importance of some other mechanism in the maintenance of CR against *C. difficile* besides the effect of antimicrobial substances. Studies with continuous flow cultures suggest that competition for nutrients, especially amino acids, may be an important mechanism in CR (Wilson and Perini, 1988; Yamamoto-Osaki *et al.*, 1994). However, continuous flow cultures are extremely dependent on culturing and incubation parameters, and their applicability to the gut environment is unclear (McFarland, 2000).

Since adhesion of a pathogen is essential for colonization and expression of virulence, the competition for mucosal receptors and inhibition of adhesion of *C. difficile* by IMF could be one mechanism of CR. Some experiments have shown that lactobacilli can inhibit adhesion of several other enteric pathogens (Coconnier *et al.*, 1993; Forestier *et al.*, 2001; Mack *et al.*, 1999). Specific carbohydrates decorating the cell wall of different species of lactobacilli, revealed by lectin typing (Annuk *et al.*, 2001), suggests the possibility of blocking the adhesive sites of pathogens by co-adhesion with lactobacilli. Unfortunately, there are no data on how lactobacilli or other members of IMF influence *C. difficile* adhesion. Since lactobacilli and other probiotic bacteria are frequently administrated in dairy products and sometimes combined with prebiotics, we have investigated the possible effects of these additives to *C. difficile* adhesion. We found that xylitol (as a likely prebiotic), bovine colostrums and milk whey can inhibit *C. difficile* adhesion to Caco-2 cells (Naaber *et al.*, 1996), showing the potential for designing and applying appropriate functional foods for the prevention of *C. difficile* colonization.

C. Animal Models

Several attempts have been made to reconstitute resistance to *C. difficile* infection in animals by using fecal homogenates from healthy normal animals of the same or different species or from

humans. In most of these studies with different germ-free animals, the administered complete fecal flora provided resistance to *C. difficile* (Itoh *et al.*, 1987; Wilson *et al.*, 1981, 1986). In contrast to these studies, some attempts to use a particular anaerobe or combinations for reconstituting CR against *C. difficile* have been unsuccessful (Borriello, 1990; Wilson *et al.*, 1986). However, there has been described a trixenic mouse model colonized with *Clostridium indolis, Clostridium cocleatum* and a fusiform flagellate *Eubacterium* sp. capable of inhibiting the implantation of *C. difficile* (Boureau *et al.*, 1989). In a recent experiment with this model (Thomas *et al.*, 2002) the barrier mechanism of the protective flora was assessed, focusing on the interactions taking place in the cecal mucus layer and inside of crypts. Modern molecular methods (FISH) combined with scanning electron microscopy showed that the three barrier species with mucus degrading ability, and not *C. difficile,* were embedded in the mucus layer of caecum. The tissue association of the *C. difficile* strain was 10-fold lower than that of the flagellate, showing that adhesion, deep mucus colonization and crypt association are the mechanisms responsible for the barrier effect. The challenge of animal studies is the different microbial ecology of the gut from humans and the possibility to draw only indirect conclusions.

A few animal experiments have been performed to study the role of lactobacilli in the maintenance of stability of intestinal IMF after exposure to *C. difficile*. Itoh *et al.* (1987) found that *C. difficile* overgrowth was associated with a decrease of intestinal lactobacilli in an ampicillin compromized mouse model. However, a mixture of three strains of lactobacilli together with other intestinal bacteria did not eliminate *C. difficile* in gnotobiotic mice in their experiment. Moreover, Wong *et al.* (1996) showed that human strains of *Lactobacillus* sp. fed to mice usually did not survive well, and the metabolism of introduced strains was significantly lowered. We have used a cefoxitin compromized mouse model to study changes in intestinal microflora and CR against *C. difficile* (Naaber *et al.*, 1995). Although administration of cefoxitin did not change the total counts of intestinal lactobacilli, mice became more susceptible for colonization by *C. difficile*. This colonization was short-term and no real infection developed. However, in these experiments detection of just total counts of lactobacilli could miss changes in the species composition of lactoflora and was not able to track the highly antagonistic strains.

Since in most experiments probiotic bacteria alone fail to protect against *C. difficile* infection (Borriello, 1990), we have tried the combination of prebiotic together with probiotic. Administration of

Lactobacillus GG together with xylitol prevented lethal *C. difficile* infection in 4/5 of hamsters (Naaber *et al.*, 1998a). *Lactobacillus* GG alone failed to protect these animals. Mechanisms of protective effect of xylitol could be inhibition of adhesion of *C. difficile* and promotion of growth of lactic acid bacteria in the gut (Naaber *et al.*, 1996; Salminen *et al.*, 1985).

Considering the results of *in vitro* experiments and animal models, we can conclude: (1) antimicrobial activity of lactobacilli and susceptibility of *C. difficile* to this antagonistic activity varies in different strains; (2) there is some association between the counts of intestinal lactobacilli and CR against *C. difficile,* but the exact role of lactobacilli and the mechanism of action are not clear; and (3) according to animal models, however, it seems that lactobacilli are not the only group of bacteria responsible for the protection against AAD and CDAD. The dramatically different intestinal flora of rodents and guinea pigs may also be the reason for some failures.

V. Use of Biotherapeutic Agents in Clinical Studies

Standard treatment of CDAD includes oral administration of vancomycin or metronidazole for 7 to 10 days (Pothoulakis and LaMont, 1993; Tabaqchali and Jumaa, 1995). Although definite improvement is usually noted just 3–4 days after such treatment, one or multiple serial relapses of CDAD can occur in 10 to 20% of patients (Fekety and Shah, 1993). For other AAD that are not caused by *C. difficile,* no specific antimicrobial treatment is recommended. Therefore, new strategies for treatment and prophylaxis of CDAD and other AAD that restore intestinal IMF and improve CR to *C. difficile* have been explored extensively. The most natural, and in animal experiments, successful approach is administration of the whole intestinal microflora of healthy persons. There are several reports about treatment of patients with relapsing CDAD by using rectal infusion of normal feces or a mixture of intestinal bacteria (Schwan *et al.*, 1984; Tvede and Rask-Madsen, 1989). Although some of these attempts were successful, there are serious ethical and practical problems with this kind of treatment. Therefore, biotherapeutic strains with known safety and properties are preferred.

A. Probiotics, Prebiotics, and Synbiotics

Preparations that have beneficial effects on human health by modulation or reparation of IMF include probiotics, prebiotics and synbiotics. Oral probiotics can be defined as living microorganisms,

which upon ingestion in certain numbers exert health benefits beyond inherent basic nutrition by improving the intestinal microbial balance (Fuller, 1989; Salminen, 2001). A prebiotic is a non-digestible food ingredient that can improve the host's health by selectively stimulating the growth and/or activity of bacteria in the colon (Gibson and Roberfroid, 1995) Synergistic combinations of prebiotics and probiotics are called *synbiotics*.

Probiotic products currently on the market may be presented in the form of powders, tablets or capsules, liquid suspensions, or sprays. The main branch of the probiotics industry entails preparations specifically designed for carriage of particular probiotic strains such as yogurts and different fermented foods, including cheeses. The European probiotic yogurt market is very fast growing, grossing nearly 800 million EURO in 1998 (Fooks *et al.*, 1999).

More commonly used probiotics contain lactic acid bacteria (lactobacilli, streptococci) and bifidobacteria. The main function of probiotics is to restore the impaired colonization resistance, which can be achieved by following the important requirements including the ability to survive transport to the active site; adhesion to the gut mucosa in the active site; ability of reproduction; ability to exist in symbiosis with mucosal biofilm; ability to exist in the presence of factors that disrupt CR.

Exact mechanisms of the protective effect of probiotic lactobacilli have not yet been fully elucidated, but several putative mechanisms have been postulated. These include direct or indirect suppression of pathogenic microorganisms in the gut, support for reestablishment of IMF and increase the defense of the host: (1) several products of lactobacilli such as short-chain fatty acids, hydrogen peroxide and bacteriocins have substantial antagonistic activity against several pathogens at least *in vitro*; (2) lactobacilli can prevent colonization of pathogens by blocking of adhesion sites on mucosa; (3) lactobacilli can block toxin receptor sites; (4) lactobacilli can suppress the overgrowth of pathogens by competition for essential nutrients; (5) lactobacilli may affect nonhumoral immunity (e.g., increase macrophage activity); and (6) lactobacilli can attenuate the virulence of pathogens (Fooks *et al.*, 1999).

In our laboratory, some additive putative mechanisms of action by lactobacilli for providing CR have been proposed. It was shown that different lactobacilli serve as antagonists to pathogens more effectively if tested either in microaerobic or anaerobic environments (Annuk *et al.*, 2003). This could drive searching for probiotics against *C. difficile* specific strains, particularly *L. acidophilus* or *L. casei* group

members predicted to be active in the cecum. In a human clinical trial we have shown that administration of probiotic lactobacilli, particularly *Lactobacillus rhamnosus* GG, increases simultaneously the population of the other IMF members (such as anaerobes) that may have an important role in the maintenance of CR (Sepp *et al.*, 1993). Recently we succeeded in showing that some probiotic strains, particularly *L. fermentum* ME-3 (DSM 14241) express substantial antioxidative activity (Kullisaar *et al.*, 2002), which can diminish the deleterious effect of excessive oxidative stress on epithelial cells during intestinal salmonellosis (Tamm *et al.*, 2002).

In animal models we have assessed the ability of lactobacilli for translocation (Mikelsaar and Türi, 1990; Naaber *et al.*, 2000) without causing infection. The same was shown by Berg (1995); in fact, the phenomenon recently has been suggested as a basis for action of lactobacilli with leukocytes subsequently entering circulation (Cross *et al.*, 2002). The components of the Gram-positive bacterial cell wall or intact bacterial cells can actively communicate with immune cells transducing the nuclear factor κB and STAT-mediated signals. The host responds to such stimuli by the release of pro- or anti-inflammatory cytokines, depending on the properties of the *Lactobacillus* strains (Maassen *et al.*, 2000; Miettinen *et al.*, 2000; Wallace *et al.*, 2003). Moreover, the beneficial effects of probiotics (LGG) on intestinal epithelial cells have been attributed to either preventing cytokine induced apoptosis (Fan Yan and Polk, 2002) or increased enterocyte production (Banasaz *et al.*, 2002). This may drive our attention to search for more specific immune-enhancing and mucosa-restoring probiotic strains against *C. difficile*.

B. Lactobacilli in Prophylaxis and Treatment of CDAD and AAD

One of the most widely used and investigated *Lactobacillus* strains with probiotic properties is the *Lactobacillus rhamnosus* strain GG (LGG). LGG is resistant to bile and low pH; it can adhere to intestinal mucosa and produce antimicrobial substances (Goldin and Gorbach, 1996; Saxelin, 1995; Silva *et al.*, 1987). This strain has been used successfully for the prevention of relapses of *C. difficile* colitis (Table II) in some earlier studies (Biller *et al.*, 1995; Gorbach *et al.*, 1987). However, the patient number in these studies was small (5 and 4). A double-blind placebo controlled trial was performed in Sweden with *Lactobacillus plantarum* 299v to prevent further recurrent episodes of CDAD (Wullt *et al.*, 2003). The recurrence of clinical symptoms (main outcome) was seen in only 4 patients of 11 who

TABLE II

Clinical Trials Using Lactobacilli in Treatment or Prevention of AAD and CDAD

Probiotic strain	Indication/Antibiotic	Number of patients	Therapeutic effect	Reference
LGG	Prevention of relapses of CDAD	5	Decreased frequency of relapses	Gorbach *et al.*, 1987
LGG	Prevention of relapses of CDAD	4	Decreased frequency of relapses	Biller *et al.*, 1995
L. plantarum	Prevention of recurrent CDAD	20	Decreased frequency of relapses	Wullt *et al.*, 2003
LGG	Treatment of AAD/Erythromycin	16	Shortened duration of AAD	Siitonen *et al.*, 1990
LGG	Prevention of AAD/various	188	Decreased frequency of AAD (17% vs. 48%)	Vanderhoof *et al.*, 1999
LGG	Prevention of AAD/various	267	No effect	Thomas *et al.*, 2001
L. acidophilus + *L. bulgaricus*	Prevention of AAD/ampicillin	98	Decreased frequency of AAD (8.3% vs. 21%)	Gotz *et al.*, 1979
L. acidophilus + *L. bulgaricus*	Prevention of AAD/neomycin	39	Decreased frequency of AAD (20% vs. 42%)	Clemens *et al.*, 1983
L. acidophilus + *L. bulgaricus*	Prevention of AAD/amoxicillin-clavualanate	27	Decreased frequency of AAD	Witsell *et al.*, 1995
L. acidophilus + *L. bulgaricus*	Prevention of AAD/amoxicillin	38	No effect	Tankanow *et al.*, 1990

received metronidazole in combination with *L. plantarum* 299v and in 6 out of 9 treated only with metronidazole in combination with a placebo. There were a relatively small number of patients as a high rate of underlying diseases obstructed inclusion and reduced compliance, yet the results encourage the performance of larger multi-centre studies for the benefits of probiotics in patients with CDAD.

Concerning AAD, administration of LGG containing yogurt shortened the duration of erythromycin induced diarrhea (2 vs. 8 days) in 16 patients compared to placebo (Siitonen *et al.*, 1990). A similar beneficial result after erythromycin-induced GI effects were obtained with *Bifidobacterium longum* (Colombel *et al.*, 1987). In another study administration of LGG resulted in a significant decrease of antibiotic associated diarrhea in 188 children (Vanderhoof *et al.*, 1999). However, a more recent randomized placebo-controlled trial did not detect any changes in the rate of antibiotic associated diarrhea in LGG treated patients (Thomas *et al.*, 2001).

In several studies a commercial probiotic containing *L. acidophilus* and *L. bulgaricus* has been used for the prevention of AAD. In most of these, administration of probiotic caused some reduction of AAD (Clemens *et al.*, 1983; Gotz *et al.*, 1979; Witsell *et al.*, 1995). However, one double-blind placebo-controlled study did not show any benefit of *L. acidophilus* + *L. bulgaricus* for the prevention of amoxicillin induced diarrhea (Tankanow *et al.*, 1990).

An interesting biotherapeutic agent is a non-pathogenic yeast, *Saccharomyces boulardii,* that was isolated in Indochina and which grows at the unusually high temperature of 37 °C (reviewed by Marchand and Vanderplaas, 2000). This agent is intrinsically resistant to antibiotics except for nystatin. The mode of action seemingly relies on antisecretory mechanisms by two proteins of *S. boulardii*. One protein, 120 kD, reduces the formation of cyclic AMP in the intestinal cells driving the enterocytes for secretory diarrhea. The second protein, 54 kD, is a protease that acts on toxin A of *C. difficile*. Several successful clinical trials against *C. difficile* relapsing colitis have been described: the mortality was decreased and the effects of toxin A and B on mucosa were inhibited (Capano *et al.*, 1998; Castagliuolo *et al.*, 1999; Castex *et al.*, 1990).

The reasons for conflicting data may be due to different samples of patients (adults vs. children), differing gastrointestinal motility (adults vs. elderly with constipation), different individual gastrointestinal microflora with different susceptibility to antibiotics, and last but not least, different types/species of probiotics used (Table III). As shown above, not all clinical strains of *C. difficile* were equally susceptible

TABLE III

PROBIOTIC *LACTOBACILLUS* SP. (LB) ACTION AGAINST *CLOSTRIDIUM DIFFICILE* (CD)

Predicted influence of LB against *C. difficile*	Hypothetical limitations
In caecum LB are among predominant populations	Geographical/ethnic/individual differences in intestinal LB composition
Indigenous LB are suppressed during antibiotic therapy	Species specific antibiotic susceptibility of LB
LB restore CR induced by antibiotic therapy	CR restoration by LB is not 100% (not in all patients)
	Even some antibiotics, e.g. metronidasole, not active against lactobacilli can induce CDAD
LB strains with antioxidative properties may defend epithelial cells in gut	Gut environment may not support LB survival (substrate deficiency, toxic compounds)

against the tested wide set of lactobacilli. Use of certain antibiotics (vancomycin) for treatment *C. difficile* infection may also suppress the probiotic strain if it belongs to the *L. acidophilus*-group. Moreover, it can be considered from clinical trials that the efficacy of one probiotic may not be the same in all patients, explainable with the above-mentioned geographical vs. ethnic vs. individual differences of GI microflora, or caused by the suitable vs. non-suitable gut environment for expression of probiotic properties (anaerobic; presence of non-absorbed prebiotic substances in colon).

VI. Conclusions and Further Perspectives

The complexity of intestinal microecosystems makes it extremely difficult to evaluate the role of some particular microorganisms in maintenance of stability of IMF and CR against pathogens. This complexity also puts limits to in vitro studies of CR and its mechanisms.

However, studies of AAD and particularly CDAD have shown that damaging of intestinal lactobacilli may play an important role in the pathogenesis of these diseases, and some lactobacilli can be successfully used for protection against CDAD and other AAD.

Until now, elaboration of effective probiotics is mainly based on empirical success or failure. Investigation of probable protective properties of lactobacilli, such as antagonistic activity against pathogens

and antioxidative capacity, can lead to the development of new combinations of individually selected pro- and prebiotics for different patients with CDAD.

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Bacterial Diversity in the Human Gut

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I. Introduction

The human digestive tract is colonized by microorganisms to varying degrees throughout its length. Because of acid pH, and the short retention time of gastric contents, numbers of viable bacterial cells in the stomach are usually $<10^2$ per ml, with aciduric Gram-positive species such as lactobacilli and streptococci predominating, although numbers can increase 1000-fold postprandially. While the rapid passage of digestive materials through the upper small bowel does not allow time for significant bacterial growth to occur, their numbers increase considerably to $>10^8$ per ml in the distal ileum (Macfarlane and Cummings, 1991). The rate of movement of intestinal contents slows markedly in the large bowel, which facilitates the development of large complex bacterial communities (Cummings, 1978; Cummings *et al.*,

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1993). Indeed, the vast majority of cells associated with the human body (eukaryotic and prokaryotic) are anaerobic bacteria growing in the colon (Savage, 1977). Bacteria comprise 40–45% of fecal material on a dry weight basis (Stephen and Cummings, 1980), which equates to about 18 grams of bacterial dry matter, or a total bacterial mass in the colon of about 90 grams (Macfarlane and Cummings, 1991).

The large bowel is the main area of permanent microbial colonization of the human gastrointestinal tract, and several hundred bacterial strains and species have been isolated from this complex ecosystem, where viable counts in feces typically reach 10^{11}–10^{12} per gram (Finegold *et al.*, 1983; Moore and Holdeman, 1974), with anaerobic bacteria predominating (Finegold *et al.*, 1974; Hentges, 1993; Holdeman *et al.*, 1976; Moore and Holdeman, 1974). Microbial cell population densities increase progressively from the cecum to the distal large intestine (Fig. 1). Bacterial colonization of the gut is affected by a wide variety of host, microbiological, environmental and dietary factors, as indicated in Table I.

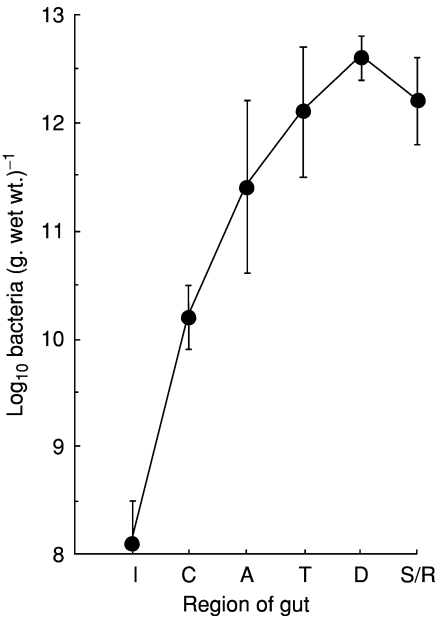

FIG. 1. Viable counts of bacteria in gut contents obtained from human sudden death victims (r = 10). I, ileum; C, cecum; A, ascending colon; T, transverse colon; D, descending colon; and S/R, sigmoid/rectum (G. T. Macfarlane, unpublished results).

TABLE I

FACTORS AFFECTING BACTERIAL DIVERSITY IN THE LARGE BOWEL

Host	Microbiological	Environmental
Diet. Host genetics	Competition for limiting nutrients and adhesion sites on food particles, mucus and intestinal mucosa. Cooperative interactions between microorganisms	Amounts and types of substrate available for growth
Colonic transit time, epithelial cell turnover rates	Bacterial genetic, biochemical and physiological traits	pH of gut contents
Disease, drugs, antibiotics, rates of mucus production and its chemical composition, pancreatic and other digestive secretions	Inhibition of invading species by metabolites produced by commensal bacteria such as sulfide, fermentation acids, phenols, deconjugated bile salts. Bacterial secretion of antagonistic substances such as bacteriocins	Redox potential
IgA and lysozyme production, and defensin secretion at mucosal surface	Bacterial secretion of antagonistic substances such as bacteriocins	Geographical and cultural factors associated with the host

Until recently, our knowledge of bacterial diversity in the large intestine was derived by using classical viable counting techniques; however, the advent of molecular methods of analysis has led to renewed interest in the structure and composition of the gut ecosystem.

II. Analysis of the Gut Microflora

A. CULTURING STUDIES

Selective and non-selective culture methods have been the standard techniques used to quantitate bacterial populations in feces; however, for various reasons, not all bacteria can be cultivated. This can result in underestimation of bacterial population sizes and microbiota diversity (O'Sullivan, 1999; Wilson and Blitchington, 1996), although culturing techniques have been thought to overestimate numbers of some important groups of gut bacteria, such as bacteroides and bifidobacteria (Dore *et al.*, 1998; Sghir *et al.*, 2000).

Methods of sampling, transport, and storage in cultivation techniques and identification vary in different studies, and this can lead to differences in results, while freezing of fecal material has been shown to significantly reduce bacterial viablity (Bonten *et al.*, 1997). In the large intestine, anaerobic bacteria outnumber aerobes and facultative anaerobes by a factor of 100–1000 (Simon and Gorbach, 1984), and this necessitates the use of anoxic conditions to grow these organisms, together with specialized protocols and apparatus, such as roll tubes, agar shakes, and anaerobic jars and chambers (Levett, 1991).

Because of the complexity of the gut ecosytem, the use of selective agars to detect all of its constituent species is a considerable task that requires large amounts of consumables, labor, and time. Moreover, selective agars are usually not entirely selective, while some species are able to grow better than others. Some bacteria have complex nutritional requirements that are not met with synthetic culture media, or the organisms are obligate syntrophs that will not grow in isolation and require the metabolic activities or products of other species for their existence (Macfarlane and Gibson, 1994; Macfarlane *et al.*, 1994). For example, methanogenic organisms such as *Methanobrevibacter smithii* are extremely oxygen sensitive, obligate hydroger-requiring syntrophs that have been found to constitute up to 12% of the culturable microflora and reach counts of 10^{11} in some individuals (Miller *et al.*, 1982; Nottingham and Hungate, 1986).

Once isolated, bacteria need to be identified by the use of biochemical tests (Holdeman *et al.*, 1977), chemotaxonomic, molecular or immunological analyses (Levett, 1991), which can be time consuming and may be dependent on a particular stage of growth of the organism. This may also limit the number of fecal samples that can be used in an investigation and usually confines identification to the predominant species in the microbiota.

Moore and Holdeman (1974) investigated fecal populations in 20 Japanese-Hawaiians and found 113 different types of bacteria, with over 100 isolates being identified. Each person was found to harbor approximately 10–30 different species. Finegold *et al.* (1974) reported that there were approximately 30–40 major species in the fecal microflora, while a subsequent study by Simon and Gorbach (1984) found that each individual has about 40 predominant species, that constitute approximately 99% of all fecal isolates. The results of Finegold *et al.* (1983), in a study involving 141 people, demonstrated that *Bacteroides* was the predominant genus in all stool samples, with a mean count of about 10^{11} per gram of feces, as shown in Table II.

TABLE II

MAJOR BACTERIAL GENERA ISOLATED FROM ADULT FECES BY CULTURING METHODS

Bacterial group	% Specimens positive	Numbers (log_{10}/gram)
Bacteroides	99	11.3
Eubacteria	94	10.7
Anaerobic Gram-positive cocci	94	10.7
Bifidobacteria	74	10.2
Clostridia	100	9.8
Actinomyces	8	9.2
Lactobacilli	78	9.6
Streptococci	99	8.9
Fusobacteria	18	8.4

Adapted from Finegold *et al.* (1983).

B. MOLECULAR ANALYSES

Many bacteria in the large gut have been reported to be ''unculturable'' (Langendijk *et al.*, 1995; Sghir *et al.*, 2000), although to some extent this might be a reflection of the isolation methods employed, and ''difficult to culture'' might be a more appropriate way of viewing these organisms. In any case, studies comparing DNA sequences obtained from the environment to databases of known cultured bacteria indicated that many of the new sequences were not accounted for and that diversity had been underestimated (Amann *et al.*, 1995).

Comparing the DNA stain DAPI (4′, 6′-diaminido-2-phenylindole) used for total bacterial counts under the microscope, with the proportion of the microflora cultured has indicated that only about 40–60% of bacteria are recoverable in a fecal sample (Tannock *et al.*, 2000; Wilson and Blitchington, 1996). The need to identify ''unculturable'' bacteria in the gut, and the desire to have high-throughput analysis, has led to the development of many new molecular techniques (Akkermans *et al.*, 2000; Vaughan *et al.*, 2000) to study the colonic microbiota in a culture-independent way, which allows detection and in some cases quantitation of bacteria previously not found on primary isolation plates (Dore *et al.*, 1998; Franks *et al.*, 1998; Harmsen *et al.*, 2000b; Marteau *et al.*, 2001; Seksik *et al.*, 2003; Sghir *et al.*, 2000).

These methods are based on hybridizations or analyses of 16S rRNA, which contains conserved and hypervariable regions (Woese, 1987) that allow phylogenetic typing (Amann *et al.*, 1995). In these protocols,

a new species is determined as one that has a sequence similarity of less than 97% with an organism in the database (Raskin *et al.*, 1997; Stackebrandt and Goebel, 1994).

III. PCR-Based Molecular Techniques

Polymerase chain reaction (PCR) amplification is a rapid, accurate and sensitive method of analysis that can be used to detect bacteria in complex communities. It has the ability to amplify DNA fragments from a background of other genomes by the use of genus- or species-specific primers (Matsuki *et al.*, 1999; Wang, 1996) and can identify the bacteria without the need for culture. PCR can reduce the selection bias introduced by traditional culture methods (Head *et al.*, 1998), but DNA/RNA may not be extracted with equal efficiencies from all bacteria, and PCR amplification may introduce artefacts, while organisms that are more subsceptible to lysis, amplification and permeability will be detected more readily (Reysenbach *et al.*, 1992; Welling *et al.*, 1997).

Techniques used in bacterial DNA isolation can also affect the outcome of subsequent analytical procedures, because bacteria have different susceptibilities to cell lysis. Zoetendal *et al.* (2001) looked at seven bacterial species from stools and found differences in detection that were dependent on the method of lysis. For example, *Fusobacterium praunitzii* was not found by using Triton X-100, and the use of bead beating in the isolation of DNA resulted in a wider range of bacteria being detected.

The design and deployment of carefully evaluated primers can be used to identify bacteria in complex microbial communities (Alm *et al.*, 1996). Species specific primers are available for a large number of organisms, including some of the major culturable groups in feces such as bifidobacteria (Matsuki *et al.*, 1999), lactobacilli (Walter *et al.*, 2000), ruminococcus (Wang *et al.*, 1997), eubacteria (Kageyama and Benno, 2001), bacteroides (Wang *et al.*, 1996) and clostridia (Wang *et al.*, 1996).

A. 16S rDNA Clone Libraries

16S rDNA cloning and sequencing for phylogenetic analysis of the intestinal microflora has indicated the presence of hitherto unrecognized species in fecal material (Suau *et al.*, 1999; Wilson and Blitchington, 1996). Sequences of 16S rRNA are determined by creating rDNA clone libraries and comparing the sequences to databases such as Genbank and Ribosomal Database Project (RDP), which have more than 10,000

small subunit rRNA sequences. However, some of the studies done to date are limited in that they have only looked at the microflora of one individual and used partial sequences.

Suau *et al.* (1999) looked at a single stool sample from one adult male, and obtained 284 clones. It was reported that 95% of the clones could be placed in three phylogenetic groupings (*Bacteroides* group, *Clostridium coccoides* group, *C. leptum* subgroup) and that only 25% of the clones analyzed corresponded to cultivated bacteria, the rest were uncultivable. Certain organisms were found to be underepresented in the clone library, and no bifidobacteria were found, yet another method of molecular analysis with dot blots revealed the presence of bifidobacteria (Section IV.A). Wilson and Blitchington (1996) also found that the majority of clones from a human fecal sample belonged to these three phylogenetic groups, and could also find no bifidobacteria.

Hayashi *et al.* (2002) investigated three healthy subjects by making 16S rDNA clone libraries with a universal primer set and partial sequencing of randomly selected clones. They also did culturing and partial sequencing of the isolates. It was found that marked interindividual differences occurred in the gut microflora; 25% belonged to 31 known species and 75% were novel phylotypes that could not be attributed to sequences in the databases. The number of PCR cycles used to amplify 16S rDNA can also influence the type of clone library obtained, and increased cycles have been reported to result in reduced diversity (Bonnet *et al.*, 2002).

Cloning approaches are time consuming and expensive and are unable to characterize complex bacterial populations in the gut; however, by using PCR and fingerprinting methods such as DGGE (density gradient gel electrophoresis) and TGGE (temperature gradient gel electrophoresis), microbial diversity and changes in complex communites can begin to be analyzed (Zoetendal *et al.*, 1998).

B. Molecular Fingerprinting

DGGE and TGGE are molecular fingerprinting techniques that can be used for rapid population analysis in the gut that are based on 16S rDNA sequence diversity (Akkermans *et al.*, 2000; Konstantinov *et al.*, 2002; Tannock *et al.*, 1999). Nucleic acid is extracted from samples, and after amplification by PCR of 16S rRNA genes, using group-or species-specific primers, the products are then separated on a gel. A GC-clamp is added to the 5′ end of one of the primers, and this is then incorporated into the amplicons during PCR, which prevents their complete denaturation. The electrophoretic patterns on the gels can

be complex and require numerical or sequences analysis, although bands in identical positions do not always contain the same DNA.

Although at best semi-quantitative, these techniques have allowed monitoring of bacterial populations in different regions of the gut (Zoetendal *et al.*, 1998) and investigations on the establishment and persistance of probiotic bifidobacteria in feeding trials (Satokari *et al.*, 2001b). DGGE has been be used to study changes in bacterial populations that occur in low numbers in the bowel, such as lactobacilli. Using species-specific primers, it was found that successional changes occurred in lactobacilli communities in infants in the first 5 months of life (Heilig *et al.*, 2002). Akkermans *et al.* (1999), using DGGE together with cloning and sequencing of prominent bands, observed that host genetic factors affect the composition of the gut microbiota. They reported a higher similarity of banding patterns in fecal samples of genetically related compared with genetically unrelated adults and that the patterns differed from other hosts such as pigs and cats.

The limitations of DGGE are that false bands can occur because of the formation of heteroduplexes and that DNA fragments with different sequences may migrate to the same position, while sub-dominant species may not be detected.

Terminal restriction fragment length polymorphism (T-RFLP) analysis is another molecular fingerprinting technique that has been used to study gut microorganisms in pigs and rats (Kaplan *et al.*, 2001; Leser *et al.*, 2002), in which one of the PCR primers is labeled with a fluorescent dye to allow detection of the PCR product. This is then digested with restriction enzymes and the products analyzed with a DNA sequencer. The terminal restriction fragments can then be compared to sequences in the Ribosomal Database Project (RDP) online website. However, the primer-enzyme combinations used have been found to be unsuitable for amplification of 16S rRNA genes from some species of *Bifidobacterium* (Liu *et al.*, 1997; Suau *et al.*, 1999). In a study by Nagashima *et al.* (2003), bifidobacteria were detected in human fecal samples from eight individuals of different ages by constructing new primer-enzyme combinations; cloning and sequencing of the terminal restriction fragments obtained showed over 99% similarity to bifidobacteria.

IV. PCR-Independent Molecular Methods

Two molecular methods can be used to quantitate bacteria by using 16S rRNA targeted oligonucleotide probes, dot-blot hybridization, and fluorescent *in situ* hybrization (FISH).

A. Dot-Blot Hybridization

In this procedure, RNA is isolated from a sample and blotted onto membranes (Dore *et al.*, 1998; Hopkins *et al.*, 2001; Sharp and Macfarlane, 2000; Sghir *et al.*, 2000). The membrane can then be hybridized with specific radioactively labeled oligonucleotide probes for bacteria and compared with a membrane hybridized with a eubacterial probe for all bacteria. The relative abundance of specific groups of bacteria in the total population can then be determined. Because individual bacterial species have different ribosome contents ranging from 10^3 to 10^5 ribosomes per cell, and the ribosome content can also vary with growth rate, the results cannot be directly converted to cell numbers (Amann *et al.*, 1995).

Sghir *et al.* (2000) used a set of six oligonucleotide probes to investigate fecal samples from 27 adults. The numerically predominant organisms were found to belong to the bacteroides group (37%), the *C. leptum* sub-group (16%), and the *C. coccoides* group (14%). Bifidobacteria, lactobacilli, and enterobacteria accounted for less than 2% of the microbiota. In another study using stools from 10 healthy adults, it was observed that 92% of all bacterial rRNA could be accounted for, with bacteroides being the predominant group, comprising 36% of the total microflora. Enterobacteria, lactobacilli, the *C. leptum* subgroup, *C. coccoides* group, and bifidobacteria accounted for 3%, <1%, 30%, 20%, and 4% of the total rRNA, respectively (Dore *et al.*, 1998).

B. Fluorescent *in situ* Hybridization (FISH)

In FISH, bacteria are usually quantitated by using oligonucleotide probes against intact bacterial cells, without extraction of RNA, and labeled with fluorescent dyes such as FITC, cy5, or cy3. The cells are treated with paraformaldehyde or ethanol to permeabilize them and allow the probe to enter, which can then bind to its target RNA and cause it to fluoresce. The numbers of target organisms are determined as a proportion of the total bacteria stained with the DNA stain 4′, 6-diamidino-2-phenylindole (DAPI) or by the eubacterial probe EUB 338, which should detect all eubacteria. The organisms can then be counted by using a fluorescent microscope.

One advantage of this procedure is that the bacteria can be detected *in situ*, which allows determination of the spatial organization of communities in the gut (Macfarlane and Macfarlane, 2003; Macfarlane *et al.*, 2000). Automation of FISH and combination with image analysis with computer software can increase sample throughput (Jansen *et al.*,

1999); however, the lower level of sensitivity is about 10^6 cells per gram of fecal material, and some bacteria may not be detected because of their impermeability, lack of target site for the universal probe, or requirements for pretreatment with lysozyme.

Species specific probes are available for some bacteria (Schwiertz *et al.*, 2000; Yamamoto *et al.*, 1992), while other species are currently undetectable. The majority of probes cover groups, such as the *E. rectale/C. coccoides* oligonucleotide probe, which has been reported to hybridize with approximately one third of the total fecal microflora (Franks *et al.*, 1998). Difficulties lie in developing specific probes for species of related genera such as *Eubacterium* and *Clostridium* (Collins *et al.*, 1994), and probes for a particular group of organisms may also hybridize with unrelated species (Kaufmann *et al.*, 1997; Langendijk *et al.*, 1995; Manz *et al.*, 1996).

A large number of probes are currently available for monitoring the presence of bacteria in the intestine that are thought to cover 80% of the resident population (Franks *et al.*, 1998; Harmsen *et al.*, 2002). Harmsen *et al.* (2002) used a set of 15 probes to look at fecal composition in healthy volunteers and were able to detect about 90% of the microorganims as compared with the universal bacterial probe and 56% as compared with DAPI (Table III).

Rigottier-Gois *et al.* (2003) developed five species-specific probes in a study combining FISH and flow cytometry of fecal samples from 20

TABLE III

QUANTITATION OF FECAL BACTERIA BY FLUORESCENT *IN SITU* HYBRIDIZATION

Bacteria	% Microbiota by Bact 338
Bacteroides/Prevotella group	28
Eubacterium rectale/Clostridium coccoides group	23
Eubacterium low G + C2	11
Atopobium group	12
Ruminococcus group	10
Bifidobacterium genus	5
Eubacterium cylindroides group	1
Enterobacteria	0.2
Veillonella	0.08
Lactobacillus/Enterococcus group	0.01

Adapted from Harmsen *et al.* (2002).

healthy adults and compared the results with culture. The results showed that bacteroides were predominant members of the gut microflora. *Bacteroides fragilis* was detected most frequently by culture and FISH analysis; however, *B. vulgatus* was found to be the most abundant bacteroides by FISH and *B. fragilis* by culture. Zoetendal *et al.* (2002b) used a combination of FISH and flow cytometry to quantitate *Ruminococcus obeum*-like bacteria and observed that these organisms were numerically important in feces, constituting 16% of the *E. rectale/C. coccoides* group.

Franks *et al.* (1998) used a suite of six probes to study fecal samples from adults over a period of eight months, and noted that bacteroides accounted for 20% of the microflora, while the *E. rectale/C. coccoides* group constituted 29% and bifidobacteria 3% of the microbiota. Bifidobacteria were found to comprise about 10% of the gut microflora when using culturing methods (Finegold *et al.*, 1974); however, another study using FISH also found that bifidobacteria numbers were lower than those determined by culture (Langendijk *et al.*, 1995).

Limitations of FISH include problems with cell wall permeability (Bidnenko *et al.*, 1998), low ribosome content, and differences in the abilities of probes to reach target sites (Fuchs *et al.*, 1998). One method of increasing acessibility is to use helper oligonucleotides that bind adjacent to the probe target site (Fuchs *et al.*, 2000). The Eub338 probe does not detect members of the Archaea or Eukarya, such as methanogens (Lin and Miller, 1998) and organisms belonging to the planctomycetales and verrucomicrobia (Daims *et al.*, 1999).

C. Comparison of Hybridization Methods for Measuring Diversity

Comparisons of studies looking at adult fecal material with various molecular techniques have shown that different results are obtained. FISH analysis with six probes (Franks *et al.*, 1998) found that bacteroides accounted for 20% of the total population, with the *E. rectale/C. coccoides* group, the *C. leptum* subgroup, and bifidobacteria constituting 29%, 12%, and 3%, respectively.

However, when using dot blot hybrizations, it was observed that bacteroides constituted 37% (Sghir *et al.*, 2000), but a broader probe was used than in the FISH study, which covered prevotella and porphyromonas as well as bacteroides. For the *C. coccoides* group, the same probe was used and accounted for 16%, while the *C. leptum* subgroup constituted 14%, and bifidobacteria constituted less than 1% of the total population. Although a different person was used in each of these hybridization studies, the difference when using the same

TABLE IV

COMPARISON OF FECAL BACTERIAL POPULATIONS USING 16S rRNA OLIGONUCLEOTIDE PROBES

	% Eubacterial probe	
	Dot blot hybridization*	FISH†
Bacteroides/Prevotella/Poryphromonas group	37	20
Clostridium coccoides group	16	29
Clostridium leptum sub-group	14	12
Bifidobacteria	1	3

*Adapted from Sghir *et al.* (2000).
†Adapted from Frank *et al.* (1998).

probe for the *C. coccoides* group indicates the difficulties in comparing molecular studies and that measuring cell numbers and abundance of ribosomal RNA using the same probe does not always give the same result, as shown in Table IV.

V. Advances in Molecular Analysis to Study the Gut Microflora

A. REAL-TIME PCR

Coventional PCR of microbial populations determines only that bacteria are present in a community and not their relative abundances. However, with the advent of real-time PCR, 16S rRNA gene copy numbers can be determined by using genus- or species-specific primers, with external standards, with the incorporation of a fluorescent dye such as SYBR green into double-stranded DNA. Real-time PCR has been used to study intestinal populations (Huijsdens *et al.*, 2002), while Malinen *et al.* (2003) employed real-time PCR and compared the detection of five fecal bacteria with dot-blot hybridization and found that the former technique was faster and more sensitive. Other studies in which bifidobacteria were investigated with culture and real-time PCR showed that both procedures had a high degree of correlation (Requena *et al.*, 2002).

Real-time PCR has advantages over older techniques such as dot-blot analysis because of its increased sensitivity and higher sample throughput. It is also quantitative in that 16S rRNA gene numbers can be related to bacterial cell counts, while there is no requirement for a radioactive label.

B. Microarray Analysis

With the application of high-throughput tools such as DNA microarrays, which can analyze RNA abundance and gene homology by coating a chip with DNA fragments, studies on genetic variations in microbial communities and differences in functional genes can be undertaken in a single experiment. With microarrays, thousands of genes can be tested on one slide; however, the equipment needed for this technology is expensive. One drawback of FISH is that a limited number of probes can be used at one time; with DNA microarrays, multiple probe sets can be employed.

Wang *et al.* (2002) developed a lower-cost membrane-array method by using oligonucleotide probes to 20 predominant intestinal bacteria. The 40-mer oligonucleotide probes were applied to a nitocellulose membrane. Extracted bacterial DNA was amplified by PCR with universal primers, labelled with digoxigenin, and then hybridized to the membrane, and a positive signal was detected by color development. The two universal primers used were able to amplify full size 16S rDNA from all of the bacteria, and the technique was able to reliably identify all 20 organisms.

VI. The Developing Microflora in Infants

Babies are colonized with bacteria from the mother and environment at birth. Whether the infant is breast or bottle-fed has an important effect on the types of bacteria that grow in the gut. While some workers have found no differences in fecal microfloras in breast and bottle-fed infants (Heavey and Rowland, 1999; Lundequist *et al.* 1985), the majority of studies have shown that there are major differences in bacterial community structure (Beerens *et al.*, 1980; Benno and Mitsuoka, 1984; Harmsen *et al.*, 2000a; Martin *et al.*, 2000).

Using culture-based methods, Benno *et al.* (1984) investigated 35 breast and bottle-fed infants and found that bifidobacteria were predominant in both groups, with *Bif. breve* being the most common isolate in both cohorts. However, counts of most of the other bacteria were higher in the bottle-fed infants (Fig. 2). In these children, the microbiota has been found to be more diversified and to more closely resemble the adult microflora. The major genera found are *Bifidobacterium, Bacteroides*, and *Clostridium*, together with facultative anaerobes (Benno *et al.*, 1984; Fuller *et al.*, 1991; Hopkins *et al.*, 2001; Stark and Lee, 1982).

Bacterial populations in the infant colon progressively change from primary colonizing facultative anaerobes, such as *E. coli* and

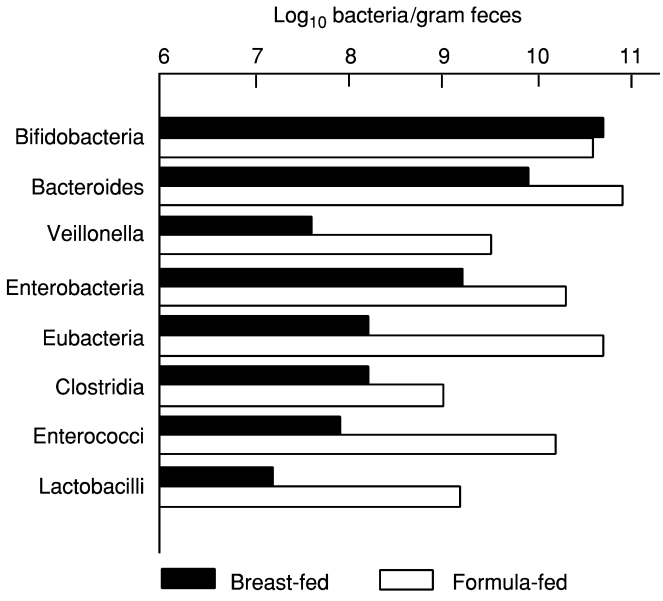

FIG. 2. Bacterial populations in breast and bottle-fed infants. Results are adapted from Beerens *et al.* (1980) and Benno *et al.* (1984).

enterococci, to communities that contain increasingly diverse assemblages of strict anaerobes, until by about 2 years of age when they begin to resemble adult-like microbiotas. Culturing studies have indicated that anaerobes are detectable in feces within 2 days of birth and that infants are colonized by a wide variety of anaerobic bacteria by day seven (Long and Swenson, 1977), when using FISH analysis, anaerobes have also been detected in fecal material on the second day after birth (Harmsen *et al.*, 2000a).

DGGE with PCR and 16S rRNA gene sequence analysis of the bands was used to follow the establishment of bacterial populations in two babies over 10 months, in a study that showed that bacterial diversity increased over time (Favier *et al.*, 2002). In this work, 19 of 34 cloned sequences had less than 97% identity with bacteria in databases. Of interest was the observation that the dominant colonizing species was a clostridium in one baby. It was also found that bacteroides did not appear to be part of the dominant microflora in these infants, which was thought to have been due to their sequences forming diffuse bands in DGGE, or that their numbers were below the technique's detection

limit. Ruminococcus bands were also found, which dominated in the babies microbiotas, and these bacteria have only been found in low to moderate numbers in culturing studies (Mackie *et al.*, 1999).

The *Coriobacterium* group can be cultured from human feces (Holdeman *et al.*, 1976); however, they have been reported to be underestimated in clone libraries (Suau *et al.*, 1999; Wilson and Blitchington, 1996), which may be due their to high G + C content inhibiting amplification. Harmsen *et al.* (2000c) developed oligonucleotide probes to target this group and the atopobium cluster, which includes the *Coriobacterium* group, and used them for FISH analysis of fecal samples from breast and formula-fed infants. The findings of the study indicated that formula-fed babies had higher numbers and greater prevalence of coriobacteria in their stools. Large numbers of these organisms were found, reaching up to 39% of total bacteria in fecal samples, which also hybridized with the *Atopobium* probe, while bifidobacteria were found in lower numbers in formula-fed babies.

Using species-specific PCR to look at bifidobacteria in adult and breast-fed infant stools, Matsuki *et al.* (1999) found that *Bif. catenulatum* was the commonest isolate in adults, followed by *Bif. longum* and *Bif. adolescentis. Bif. breve, Bif. infantis*, and *Bif. longum* mainly occurred in the intestinal tracts of infants, and the distribution of these organisms was similar to that found in culturing experiments (Benno *et al.*, 1995). Other workers, using culturing methods, have reported that *Bif. longum, Bif. adolescentis, Bif. pseudocatenulatum*, and *Bif. parabifidum* occur in high numbers in breast-fed children (Kleessen *et al.*, 1995). In Japan, studies on infant stools have suggested that *Bif. breve* and *Bif. infantis* are the numerically important bifidobacteria. Karvonen *et al.* (1998) demonstrated that the vaginal flora of the mother had the same composition as neonates' stools; however, *Bif. infantis*, which is generally the commonest isolate from infant feces, was not found.

One explanation for why breast-fed children have intestinal bifidobacterial populations distinct from those in bottle-fed babies is that many different microbiologically active substances are present in human milk, such as glycoproteins and glycolipids, fucose, neuraminic acid, lactose, N-acetylglucosamine, a variety of oligosaccharides based on lactose (Miller *et al.*, 1994), as well as bifidogenic nucleotides (Gil and Rueda, 2000). These compounds are not assimilated to a significant extent in the infant small intestine, which has led to the suggestion that they are selectively utilized as carbon and energy sources by bifidobacteria in the large bowel (Brand Miller *et al.*, 1995).

VII. The Gut Adult Microbiota

A. The Gut Microflora in Adults

Studies investigating the stability of the adult fecal microflora by using culturing methods indicate that while bacterial groups such as lactobacilli, bacteroides, and bifidobacteria seem to remain relatively constant (Bornside, 1978; Finegold *et al.*, 1983; Holdeman *et al.*, 1976), there can be considerable changes in microbiota composition at the species level (Holdeman *et al.*, 1976). For example, in 10 volunteers studied over 12 months, numbers of *Bacteroides fragilis* were found to vary as much as a 1000-fold, although overall, bacteroides appeared stable at the genus level (Meijer-Severs and van Santen, 1986).

Culturing studies suggest that within an individual, the predominant species do not change to any great extent unless perturbed by variations in diet, disease, or antibiotic treatment (Moore and Moore, 1995; Simon and Gorbach, 1984). With molecular fingerprinting, it has been shown that antibiotic therapy, or the introduction of an allochthonous microorganism such as a probiotic, can affect bacterial diversity in the gut (Satokari *et al.*, 2001b; Tannock *et al.*, 2000). Some organisms are able to persist in the gut and become dominant populations, while in contrast other species are transient and may only be detectable for a few days.

Molecular fingerprinting techniques also suggest that while the composition of the microbiota is comparatively stable after weaning, it appears to be individually unique and is influenced by host genetic factors (Van de Merwe *et al.*, 1983; Zoetendal *et al.*, 2001).

Populations of the predominant bifidobacterial strains in the gut are also stable, and studies show that each person harbors at least one specific bifidobacterium or lactobacillus strain (Kimura *et al.*, 1997). Some culturing studies have shown that *Bif. adolescentis* is the most common bifidobacterium in adults (Mutai *et al.*, 1987), however, with PCR, *Bif. catenulatum* and *Bif. longum* were found more often (Matsuki *et al.*, 1999). Satokari *et al.* (2001a) found that using PCR with bifidobacterial-specific primers and DGGE, bifidobacteria were unique in 5 adults and that bifidobacteria populations were host-specific and stable over a period of 4 weeks. Other workers have also shown bifidobacteria to be host specific (Mangin *et al.* 1999; McCartney *et al.* 1996). It has also been observed that in the adult intestine, there is usually a combination of one to four species (Mangin *et al.* 1999; Matsuki *et al.*, 1999). It should be noted that these apparent differences in gut bifidobacteria are probably, to some degree, artifacts of the detection methods. For example, Matsuki *et al.* (1999) demonstrated that *Bif. adolescentis*

could be detected by PCR but not by culture and sequencing from fecal material.

B. The Aging Gut

Modifications in diet and host immune system activity and physiological changes in the digestive tract inevitably affect the composition and metabolism of the gut microflora in older people. The elderly have been shown to have fewer bifidobacteria and higher numbers of enterobacteria and clostridia than young adults (Gorbach *et al.*, 1967a; Hopkins and Macfarlane, 2002; Hopkins *et al.*, 2001; Mitsuoka *et al.*, 1982). Reductions in bifidobacteria and an increase in clostridia, *C. difficile*, and enterococci have also been found in older dogs (Benno *et al.*, 1992).

There is good evidence for reduced bacterial species diversity in stools from elderly people (Hopkins and Macfarlane, 2002), and it has been suggested that this may be due to a larger proportion of their microflora being unculturable (Korshunov *et al.*, 2001). Differences have been reported in species of bifidobacteria and enterobacteriaceae in children and the elderly, although it seems that there is a general reduction in bacterial species diversity in older people (Gavini *et al.*, 2001; Hopkins and Macfarlane, 2002; Percival *et al.*, 1996).

Changes in bifidobacteria are of particular interest because of their association with gut health, and a wide range of these organisms occur in infants and young adults. However, in the elderly population, it has been reported that species diversity is reduced to one or two dominant organisms, in particular *Bif. adolescentis* and *Bif. longum* (Gavini *et al.*, 2001; He *et al.*, 2001). It has been suggested that reducing the abilities of bifidobacteria to adhere to the colonic mucosa may be a factor in the decline of bacteria in older people (Ouwehand *et al.*, 1999).

Hopkins and Macfarlane (2002) investigated species diversity in elderly people by using culturing methods and found that bacteroides species diversity increased in the feces of elderly people, while bifidobacterial species diversity was reduced. *Bifidobacterium angulatum* was the most common isolate in young adults, and several bifidobacterial species were not detected in the elderly, including *Bif. bifidum, Bif. catenulatum, Bif. pseudocatenulatum* and *Bif. infantis*. In previous work (Hopkins *et al.*, 2001), stools from adults, children, old people, and elderly patients with *C. difficile* associated diarrhea were studied. By using viable counts, dot-blot analysis and cellular fatty acid profiles, it was found that older people had higher numbers of enterobacteria in stools and fewer anaerobes.

Combining FISH and automatic microscope image analysis, Welling *et al.* (2002) studied bifidobacteria and bacteroides in gut material from newborn babies, breast-fed infants, adults and elderly people and found large differences between young adults and older people. The respective percentages of bifidobacteria were 73, 10, 4, and 9, while bacteroides were 1, 25, 21, and 6. They also compared people in China and the Netherlands and found no significant differences in bacteroides and bifidobacteria; however, numbers of the *E. rectale/C. coccoides* group were higher in China than in the Netherlands.

Benno *et al.* (1989) compared the fecal microfloras from 15 healthy elderly people in urban and rural communities in Japan and noted that urban Japanese had less *Bif. adolescentis* but more total anaerobic bacteria, which they attributed to higher intakes of dietary fibre in the rural area.

VIII. Bacterial Colonization in Different Regions of the Large Bowel

Studies on bacterial diversity in the gastrointestinal tract are restricted to a large extent by the inaccessibility of parts of the gut. Consequently, the vast majority of studies have been made by using fecal material, which only provides information on microbial populations occurring in the lumen of the distal large intestine. This does not necessarily reflect the composition of bacterial communities in the proximal colon or on mucosal surfaces lining the bowel.

A. The Cecal Microflora

Because of practical difficulties, and ethical reasons, very few microbiobiological studies have been done on the proximal large intestine (Gorbach *et al.*, 1967b; Macfarlane *et al.*, 1992). Marteau *et al.* (2001) compared culture and dot-blot hybridizations by using six probes to investigate the microbiotas of the cecum and fecal material from 8 subjects. Samples of cecal fluid were collected via an intestinal tube. Similar results were found with both methods of analysis. *Escherichia coli*, enterococci, and lactobacilli were shown to occur in high numbers in the cecum and accounted for 50% of total bacterial RNA, yet these organisms only account for about 7% of RNA in fecal material from the same subjects. Facultative anaerobes comprised 25% of total bacterial counts in the cecum as compared with 1% in the feces, and numbers of anaerobes such as bacteroides, bifidobacteria, the *C. leptum* subgroup and the *C. coccides* group were lower in the cecum. Total anaerobic counts in the cecum were observed to be 100-fold lower than in fecal material.

B. Mucosal Populations

Mucosal bacterial communities in the large bowel are difficult to study in healthy people, and this has limited their investigation. Samples are usually obtained from diseased individuals or patients that have been pretreated with antibiotics, or the bowel has been cleansed before colonoscopy, and so bacterial populations on these tissues may not reflect the normal mucosal microflora. Despite these problems, there is evidence for independent mucosal communities in humans (Croucher *et al.*, 1983; Lee *et al.*, 1971), although some authors have reported that the composition of epithelial populations is broadly similar to those that exist in the gut lumen (Nelson and Mata, 1970). Early culturing studies found that bacteroides and fusobacteria were the predominant bacteria on the colonic wall (Croucher *et al.*, 1983), but a wide range of other organisms such as clostridia, eubacteria, and anaerobic Gram-positive cocci have also been reported (Croucher *et al.*, 1983; Edmiston *et al.*, 1982).

In a study using culturing techniques, bacteroides populations were found to differ in fecal and colonic tissue from 10 patients with colon cancer. *Bacteroides vulgatus* was the most commonly isolated species from stools and was present in lower numbers on the mucosa, where *B. fragilis* predominated (Namavar *et al.*, 1989). Poxton *et al.* (1997) studied patients with ulcerative colitis and healthy individuals and observed few differences in the bacteria isolated, while mucosal communities were found to be generally similar to those in the gut lumen. Bacteroides were reported to be the major anaerobes on epithelial surfaces in the large bowel, with *B. vulgatus* and *B. fragilis* predominating.

Hold *et al.* (2003) used cloning in studies on colonic tissue from three people, which showed that 85–89% of bacteria occurred in the three same phylogenetic groups found when fecal samples are cloned (Suau *et al.*, 1999), as shown in Table V. Interestingly, no bifidobacteria were detected in the latter study, which has also occurred in other cloning experiments with feces (Suau *et al.* 1999; Wilson and Blitchington, 1996). Twenty-eight percent of the sequences recovered were less than 97% related to known bacteria in databases. Zoetendal *et al.* (2002a) used PCR with DGGE to compare biopsy samples from the ascending, transverse, and descending colons of 10 people as compared with fecal samples. They reported that the microflora was host specific and uniformly distributed along the colon and differed significantly from the fecal samples.

In another culturing study, bacteroides and bifidobacteria were found to be the predominant bacteria on the rectal mucosa, and

TABLE V

COMPARISON OF INTESTINAL BACTERIAL POPULATIONS FROM FECAL MATERIAL AND GUT TISSUE USING rDNA CLONING

		% Clones attributed to phylogenetic affiliations		
		C. coccoides group	*C. leptum* sub-group	*Bacteroides* group
Feces*	284 clones from one person	44	20	34
Colonic tissue†	110 clones from three tissue samples	43–49	10–18	24–35

*Adapted from Suau *et al.* (1999).
†Adapted from Hold *et al.* (2002).

bifidobacterial numbers were lower in UC patients (Macfarlane *et al.*, 2004). Other investigations, using RNA in situ hybridizations, have indicated that the mucus layer in rectal biopsies is more heavily colonized by bacteria in IBD patients, including those with ulcerative colitis (Schultsz *et al*, 1999). These studies found no bacteria in 71% of the control subjects and 32% of the IBD patients, which is surprising, since culturing studies have shown that the rectal mucosa harbors large numbers of bacteria in both health and disease (Macfarlane *et al.*, 2004; Hartley *et al.*, 1992).

Real-time PCR in conjunction with primers and probes for *E. coli* and *B. vulgatus* has been used to quantitate these bacteria on the colonic mucosa (Huijsdens *et al.*, 2002). The method proved to be very sensitive in that it was able to measure as little as 1 CFU of *E. coli* and 9 CFU of *B. vulgatus*. Counts of *E. coli* were 100-fold more than results obtained by culturing procedures on the same samples.

In the gut lumen, facultative anaerobes have been found to be outnumbered by a factor of between 100 and 1000 by strictly anaerobic species (Hopkins *et al.*, 2001). Although it has been reported that strict anaerobes were 10 to 100-fold higher than facultative anaerobes in rectal biopsies (Poxton *et al.*, 1997); however, the patients were taking antibiotics and had been prepared for colonoscopy. When bacteria were cultured from rectal biopsy tissue from healthy people and patients with ulcerative colitis who had not received antibiotics and had not been pre-treated prior to colonoscopy, it was found that strict anaerobes outnumbered facultative species by a factor of about 5–10

(Macfarlane, 2000), which was similar to values obtained from colonic tissue, at autopsy (Croucher *et al.*, 1983).

IX. Conclusions

Early studies on the composition of the human colonic microbiota depended on culturing techniques. These investigations demonstrated that the large intestine was a highly complex microbial ecosystem containing vast numbers of bacteria that could be assigned to several hundred bacterial species. However, there have been great developments in molecular methods of population analysis over the last decade, which have indicated that the colonic microbiota contains greater bacterial diversity than was previously thought. Several groups of bacteria not detected in culture have been reported to occur in high numbers in the gut. Molecular methods of analysis are beginning to supersede culturing studies, so it is worthwhile comparing these methodologies.

The advantages of culturing techniques are that they are relatively inexpensive, widely available, and quantitative. They enable physiological and biochemical studies to be done on the isolates, and if done carefully, they can provide a good indication of ecosystem complexity. Conversely, culturing is time-consuming and labor-intensive, fecal or tissue samples need to be processed immediately, isolations are dependent on the culture media and growth conditions employed, not all viable bacteria in sample can be recovered, and some organisms appear to be recalcitrant to culture. Once isolated, the bacteria have to be identified by biochemical tests, chemotaxonomic, immunological, or molecular analyses. Generally, culturing is not well suited to large scale studies on the gut.

Many molecular techniques are rapid and have the potential for high throughput analysis. Biological samples can be frozen, while DNA is easily transported between laboratories. They also allow so-called unculturable species to be detected. The disadvantages of these technologies are that they tend to be expensive (e.g., real-time PCR), while some bacteria are more susceptible to lysis than others and can therefore be detected more easily. Moreover, DNA and RNA are not extracted with equal efficiencies for all bacteria. Some molecular methods are not quantitative (DGGE, TGGE) and lack sensitivity (dot blots, FISH), while there is potential for primer bias in PCR reactions. There is also a relatively limited number of PCR primer sets and oligonucleotide probes available, some with wide specificities, though

their numbers are steadily increasing, and within the near future will probably make large scale culturing work redundant.

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Interpreting the Host-Pathogen Dialogue Through Microarrays

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I. Introduction

Advanced DNA array technology combined with the ever-expanding database of sequenced genomes from prokaryotic and eukaryotic organisms has provided an unprecedented opportunity to study biology as a dynamic system. Such holistic technologies were borne out of the appreciation that a biological system is not merely an assortment of genes and proteins, each with their own defined functionality. That is, the individual components and physicochemical interactions that define the system may not explain the functioning of the system as a whole, because of to complex protein interactions, the requirements of additional cofactors, and feedback regulation of system components. In this way, a system is more than the sum of its parts, and often the individual parts do not recapitulate the true dynamic interactions taking place. In the context of the current discussion, the ''system'' in

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question could be an infected cell or infected host tissue in which disease is taking place, and the goal is to move beyond the reductionist biological approach to understand the interactions that define a disease process in an intact animal. DNA microarray technology, the ability to sample and quantify the transcriptional response of a whole cell or system, has enabled a paradigm shift in the way in which one views hypothesis-driven science and in modelling biological systems. Since the first publication appeared describing the use of high-density DNA arrays for transcriptional profiling (Schena *et al.*, 1995), the use of microarray technology has encompassed essentially every biological discipline, including evolutionary biology, ecology, tumor biology, and immunology and is now a readily available technology for essentially any molecular biology laboratory.

Microarray technology allows the study of differential gene expression between populations by measuring the competitive hybridization of labelled populations of cDNA. Typically, mRNA is isolated from cells or tissues and reverse-transcribed into cDNA, concomitantly incorporating fluorescent dyes to distinguish between populations (Arcellana-Panlilio and Robbins, 2002). Labelled cDNAs are then hybridized to the microarray slides that contain an ordered array of cDNA elements in an addressable format. Specialized slide scanners are employed to measure the fluorescent signal intensity of individual array elements. Holloway reviews the numerous different microarray formats available to the scientist (Holloway *et al.*, 2002). Although the basic framework is the same, the specific technologies employed can vary with regard to the type of solid support, type of DNA fragment used to capture complementary cDNA, method of DNA deposition, and the detection scheme used to quantify hybridization levels on the slide. The most common frameworks are spotted glass slide microarrays, in which presynthesized DNA (usually a PCR product) is bound to a glass slide, and high-density oligonucleotide arrays in which oligonucleotides are synthesized *in situ* (Kato-Maeda *et al.*, 2001). After washing to remove non-specific hybridization, the amount of specific transcript hybridization is assayed with a confocal laser scanner that can differentiate between the two differentially labelled samples. Image-processing software has been developed to locate and quantify spots, and sophisticated algorithms have been developed to normalize signal intensities between the two populations, such that the two data sets are comparable and the expression ratios for each spot can be determined.

One field in particular that owes much to advances in microarray technology is infectious disease—in particular, the molecular biology

of pathogens. Early in the development of microarray technology it was recognized that host-pathogen interactions could be studied at the genomic level of complexity (Rappuoli, 2000), conceptually incorporating both the host and pathogen contributions to virulence in a single experiment. Virulence, or the relative capacity of a pathogen to overcome body defenses, is best studied in the context of the host, since virulence phenotypes are dependent on host factors. That is, microbial virulence is a trait that manifests only in a susceptible host and involves a dynamic interaction between pathogenic factors and host components that modify the outcome of disease (Casadevall and Pirofski, 2001, 2003). Aptly, microbial virulence studies are highly amenable to a systems-based approach with microarrays, where both elements of the host-pathogen dialogue can be tapped. In turn, by understanding the environmental cues that regulate the expression of microbial virulence genes in intact hosts, reconstruction of these environmental changes *in vitro* can help sort out the molecular basis for these virulence programs. Microarray technology has helped establish a host-pathogen lexicon that one can begin to decipher by using directed approaches involving *in vitro* and complex biological models. As the use of microarrays to study aspects of the host-pathogen interaction has become more pervasive, so too have the analytical tools and statistical methods to interpret reliably these data been refined. While the description and relative merits of these statistical methods are beyond the scope of this chapter, the reader is directed to recent seminal papers (Arfin *et al.*, 2000; Troyanskaya *et al.*, 2002; Tusher *et al.*, 2001) and reviews (Cui and Churchill, 2003; Quackenbush, 2002; Xiang *et al.*, 2003) describing refined statistical methods for microarray analysis.

In this chapter we discuss the use of DNA microarray technology to understand how microbes interact with host cells, how hosts respond to different infections, and how this dynamic interaction shapes the outcome of disease. While the literature is replete with microarray studies from the field of virology, we have chosen to pay particular attention to the area of bacterial pathogenesis. We highlight novel ways in which microarrays are being used as a modern-day translation machine to decipher the host-pathogen lexicon. After discussing examples of bacterial and host arrays, we introduce a superior detection platform that may facilitate analysis of rare cell populations or rare abundance RNA transcripts, and provide a commentary on some important technical aspects of host-pathogen interactions that may confound microarray experiments. We conclude with perhaps the most important aspect of array analysis; validation of

the results with independent methods of RNA quantitation, as well as downstream biological assays.

II. Host Arrays

Interference with host cell processes and the ensuing host response to infection govern the way in which bacteria take command of a cell. These interactions often involve activation of specific cell signaling cascades to facilitate bacterial entry and to prime the intracellular environment, making it compatible with bacterial growth, survival and evasion of host immune responses (Cornelis, 2002a,b; Finlay and Falkow, 1997; Gruenheid and Finlay, 2003; Knodler *et al.*, 2001). Some bacteria colonize their hosts by entering and surviving inside cells of myeloid and epitheliod lineages, thereby subverting host cell defence mechanisms and providing a niche to gain access to deeper tissues and organs (Rosenberger and Finlay, 2003). Therefore, research into the biochemistry of how these pathogens adhere, enter, survive, replicate within, and exit their hosts ought to take place within the cellular milieu of the host cell. Pascale Cossart and colleagues in 1996 (Cossart *et al.*, 1996) first coined the term ''cellular microbiology'' to describe a new scientific discipline bridging fundamental cell biology and microbiology. This came from the expanding appreciation that pathogenic bacteria often behaved differently when exposed to mammalian host cells than when grown in pure broth cultures. Cellular microbiology seeks to provide a deeper understanding of the evolution of pathogenicity and to understand how pathogenic bacteria can live habitually within host cells and in turn, cause disease in the host animal. Importantly, the burgeoning field of cellular microbiology attempts to define determinants of bacterial virulence in the context of the host cell, such that the lessons learned will offer a more rational approach to therapeutic intervention. Three types of experimental strategies using host arrays to study microbial pathogenesis will be discussed in this section. The first involves the use of expression profiling to determine the global transcriptional response of a host cell to a bacterial pathogen. Historically, these were the first types of host microarray studies used in microbiology research and have now been performed with a wide variety of bacterial pathogens and relevant host cells. The second strategy involves a more directed approach to investigate how specific bacterial virulence factors affect the host transcriptional program. These studies have been best tackled by using bacterial mutants deleted in certain virulence factors as compared with their isogenic wild-type parent strains. The third strategy that we

discuss is the use of host arrays to characterize the innate immune response to bacterial pathogens. These types of studies have been highly instructive in identifying not only the conserved activation programs that define what we currently know about non-specific immunity but also in highlighting pathogen-specific differences in immune cell response to different bacteria. Immunological studies of this nature are especially germane to specialized bacterial pathogens that have evolved to subvert the innate immune program and survive within professional phagocytes. Such studies emphasize how bacterial virulence mechanisms, which are often unique to a given pathogen, modify the innate response programs of professional immune cells with implications for host colonization, persistency, and chronic infections.

A. Host Transcriptional Profiling During Infection

As a logical starting point, the first reports of microarray experiments from cell culture infection models involved the use of host cell arrays to profile the hosts response to invading bacteria (Belcher *et al.*, 2000; Coombes and Mahony, 2001; Cohen *et al.*, 2000; Eckmann *et al.*, 2000; Ichikawa *et al.*, 2000; Rosenberger *et al.*, 2000) or virus (Zhu *et al.*, 1998). Arrays used in these studies were composed of human cDNAs and expressed sequence tags (ESTs) and described the host transcriptional responses to various pathogens when using *in vitro* cell culture models with relevant host cells. While these first studies were highly descriptive in nature, they were valuable as hypothesis-generating tools that could be followed up with biological experimentation.

Our laboratory was involved in the first report describing the transcriptional response of macrophages to infection with the enteric pathogen *Salmonella enterica* serovar Typhimurium (Rosenberger *et al.*, 2000). We found that *S.* Typhimurium infection caused significant changes in the expression of several genes encoding chemokines, cell surface receptors, and signaling molecules following infection of the RAW 264.7 murine macrophage cell line. An important finding from this work was an overlapping spectrum of genes expressed in response to virulent *S.* Typhimurium and purified *S.* Typhimurium lipopolysaccharide (LPS), suggesting that the early response of macrophages to bacterial infection is dominated by this generic bacterial molecule. This work also identified an intracellular signalling pathway mediated by the signalling protein MEK in response to *Salmonella* infection. This MEK kinase cascade and phagocyte NADPH oxidase activity was required to impair bacterial replication in these cells and

indicated that these two signaling pathways generate a host bacteriostatic activity that could play an important role in innate host defense against intracellular pathogens (Rosenberger and Finlay, 2002).

We have also used this strategy to characterize the mRNA expression profile of human vascular endothelial cells following infection with the obligate intracellular bacteria *Chlamydia pneumoniae* (Coombes and Mahony, 2001) in an attempt to define potential mechanisms of *C. pneumoniae* contribution to atherosclerosis and coronary artery disease. *C. pneumoniae* infection upregulated mRNA expression for approximately 8% of the arrayed genes, with the most prominently upregulated being those involved in the innate immune response such as interleukin (IL)-1, IL-8, monocyte chemotactic protein-1, and cellular growth factors (heparin-binding epidermal-like growth factor, basic fibroblast growth factor and platelet-derived growth factor B chain. These studies were highly instructive in identifying a putative biological mechanism of chronic chlamydial disease involving the elaboration of smooth muscle cell growth factors, proliferation of smooth muscle cells in the vicinity of infected tissue, and aortic intimal thickening in an intact animal host (Coombes *et al.*, 2002). However, a limitation of the foregoing studies was that they measured host cell responses to bacteria undergoing a cyclic developmental cycle—akin to acute infection with actively replicating bacteria. Importantly, a comprehensive investigation of host cell transcription in persistently infected cells has not been undertaken, but these experiments are highly justified, considering that chronicity is pathognomonic of chlamydial disease and several other important bacterial-mediated diseases such as tuberculosis. As an aside, data from chlamydial microarrays to monitor bacterial gene expression inside host cells have now appeared (Belland *et al.*, 2003; Nicholson *et al.*, 2003) (see Section III). These important studies have offered valuable information about gene expression in a bacterial system that is notoriously difficult to work with due to its obligate intracellular parasitic lifestyle and lack of a genetic transformation system.

Our array studies with endothelial cells as host targets of *C. pneumoniae* revealed a prominent proinflammatory response dominated by early activation of cytokine, chemokine, and cell signalling pathways. Other studies with non-myeloid or lymphoid cell lines—mostly epithelial cells—reveal a similar proinflammatory state upon infection with other bacterial pathogens such as *Bordetella pertussis* (Belcher *et al.*, 2000), *Pseduomonas aeruginosa* (Ichikawa *et al.*, 2000; Lory and Ichikawa, 2002), *Salmonella dublin* and *S. enterica* (Eckmann *et al.*, 2000; Rosenberger *et al.*, 2000), enteropathogenic *E. coli*

(de Grado *et al.*, 2001), and *Helicobacter pylori* (Cox *et al.*, 2001). These studies generally support the idea that epithelial cells that line mucosal surfaces are important resident sentinels involved in communicating the presence of microbes to underlying host inflammatory cells (Kagnoff and Eckmann, 1997).

Pedron *et al.* examined the kinetics of Caco-2 cell transcription in response to invasive or non-invasive strains of *Shigella flexneri* by using an Affymetrix oligonucleotide microarray (Pedron *et al.*, 2003). It was found that 240 genes common to both the invasive and non-invasive strains were induced, most notably the cytochrome P-450 family. Unique to the invasive strain was the induction of a subset of genes that involve recruitment of mucosal polymorphonuclear infiltrates. It was concluded that an increase in IL-8 production is primarily implicated in mucosal inflammation.

More recently we have explored the acute human intestinal epithelial cell response to enterohaemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC; Hardwidge *et al.*, manuscript in preparation). Caco-2 cells were grown for 3 weeks on polyester membranes such that they formed tight junctions and polarized with discrete apical and basolateral poles. Following infection of these monolayers with EPEC (2348/69), EHEC (O157:H7), and an EPEC strain deficient in the type III secretion machinery (2348/69Δ*escN*), host RNA was collected and hybridized to a glass slide microarray consisting of ~14,000 human cDNAs spotted in duplicate. The host response was assayed after 1, 2, 3, and 4 hours post-infection and compared with uninfected cells. This type of experimental design will allow us to learn: (i) the epithelial response to bacterial factors unique to both EHEC and EPEC, (ii) the host response attributable to general detection of external bacterial motifs, (iii) the kinetics of the host response when first encountering a bacterium, and (iv) the proportion of the host response that is type III secretion-dependent. Figure 1 (see color insert) demonstrates the first concept, in which the host response to infection with EHEC is compared over time to that of EPEC. We used GeneSpring microarray analysis software to plot the gene expression in Caco-2 cells infected with EHEC versus those infected in a similar fashion with EPEC, at each of the four studied time points (1–4 hours). It can be readily observed that after 1 hr of infection, a subset a genes are upregulated specifically in host cells infected with EHEC, suggesting an immediate host response to Shiga toxin and proteins co-expressed with Shiga toxin.

Host microarray studies using *in vitro* infection models have illustrated an intimate relationship between the bacteria and host during

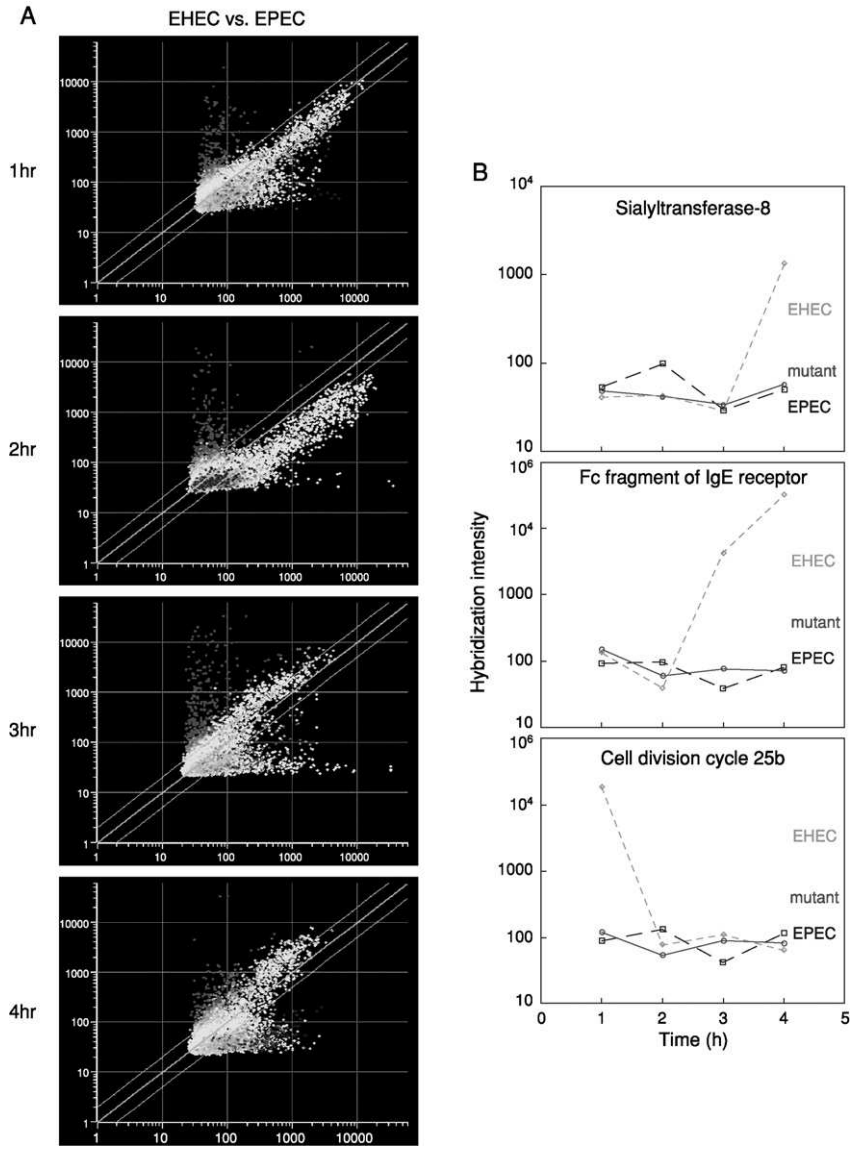

FIG. 1. Transcriptional analysis of the host response to EHEC or EPEC infection. *A*, EHEC and EPEC illicit distinct changes in host cell gene expression during acute infection. Caco-2 cell gene expression following infection with EHEC (vertical axis) or EPEC (horizontal axis), from 1 to 4 hours, is depicted graphically. Data are log-log plots of the hybridization intensity observed for each microarray spot and represent the mean of at least four independent replicates. Green lines denote gene expression values that show

infection and have illuminated the great extent to which microbial pathogenesis pervades host cell biology. Perhaps self evident, genomic data generated from such studies requires careful molecular and biochemical follow up to decipher the biological relevance of the findings, a reminder that has been discussed in the recent literature (Covacci and Rappuoli, 2003). Therefore, although these studies have intrinsic exploratory value, their real power lies in uncovering putative virulence strategies that can be followed in relevant biological systems.

B. Host Microarrays to Analyze Effector Protein Function

Gram-negative bacterial pathogens use a type III secretion system to deliver virulence proteins (called effectors) directly into host cells during infection (Blocker *et al.*, 2000, 2003; Finlay and Falkow, 1997; Hueck, 1998). While the protein structure of the type III apparatus is generally well conserved among bacterial species, the effector repertoire translocated in a type III-dependent fashion often distinguishes pathogens from one another. Once translocated, effectors modify various aspects of host biology to furnish an environment that is compatible with bacterial growth, survival, and evasion of host immunity. As such, understanding how various effectors work has been the focus of intensive research in bacterial pathogenesis. One rationale for this effort comes from the notion that unique host-pathogen interactions govern the outcome of disease following infection and that studying these unique interactions brings us closer to understanding something that might have direct clinical relevance. Microarrays have brought us closer to this understanding as valuable tools enabling the comparative analysis of the host transcriptional response following infection with virulent and avirulent mutant strains.

An interesting example of this experimental design comes from the *Yersinia* literature. The *Yersiniae* are a group of clinically important Gram-negative bacteria responsible for a spectrum of diseases ranging from gastroenteritis to plague. These bacteria are highly evolved to disseminate in a natural host and resist the innate immune response, which is linked to a virulence region contained on an extrachromosomal plasmid called pYV (Cornelis, 2000; Cornelis *et al.*, 1998). One effector encoded on pYV, called YopM (for *Yersinia o*uter *p*rotein M),

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at least a two-fold difference in either direction between samples. *B*, Representative host genes specifically upregulated by EHEC but not EPEC. Bar charts depicting differential gene expression in Caco-2 cells infected with EHEC, but not EPEC, for sialyltransferase-8 (top), the receptor for the Fc fragment of Ig-E (middle), and cell division cycle 25b (bottom).

was shown to be required for full virulence of *Yersinia* in mice (Leung *et al.*, 1990) and to transit to the host cell nucleus following injection into the host cell cytosol by extracellular *Yersinia* (Skrzypek *et al.*, 1998), suggestive of a role in altering host cell genomics. To test this hypothesis, a host microarray consisting of ~12,000 gene elements was used to profile the transcriptional response of mouse macrophages to infection with either wild-type *Y. enterocolitica* or an avirulent strain cured of pYV or a single gene mutant (*yopM*) deficient in YopM (Sauvonnet *et al.*, 2002). Following a comprehensive comparison algorithm between uninfected cells and cells infected with one of the mutant strains, it was determined that the *yopM* strain specifically regulated the expression of 25 host genes that consisted primarily of downregulated gene products involved in cell cycle regulation, DNA maintenance, and cell growth. This important study was the first to capitalize on the use of microarrays to uncover a potential biological role for a single virulence factor known to target the host cell nucleus.

In another seminal paper, Detweiler and colleagues used a 22,571 human cDNA microarray to identify host molecular pathways affected by the *Salmonella enterica phoP* gene (Detweiler *et al.*, 2001). PhoP is a stage-specific transcription factor involved in activating virulence loci required for intracellular growth and systemic disease (Hohmann *et al.*, 1996; Miller *et al.*, 1989) and also acts to repress genes that are involved in invasion of epithelial cells (Pegues *et al.*, 1995). By comparing the expression profiles of host macrophages infected with wild-type *Salmonella* and a *phoP::Tn10* isogenic mutant, the authors found that *phoP::Tn10* bacteria failed to elicit host cell induction of genes involved in cell death. The biological relevance of this phenotype was recapitulated in conventional cell biological experiments, which demonstrated that *phoP::Tn10* bacteria were defective in their ability to kill both a macrophage cell line and peripheral blood mononuclear cells. These infection-based host expression studies help to highlight the value of using arrays to first identify a putative biological mechanism, which can then be followed in cell biological experiments to uncover the relevance of the observed host response. We discuss in a later section several considerations for optimising infection parameters for microarray experiments.

C. Characterizing Innate Immunity to Pathogens

In retrospect, one of the dominant findings of early host array responses to pathogens was that there is a strong general innate immune response that significantly overlaps with many pathogens and

Pathogen Associated Molecular Patterns (PAMPs) such as LPS. For example, in early array experiments with *Salmonella* infections of cultured macrophages, there was considerable overlap in induced host genes compared to the response to LPS (Rosenberger *et al.*, 2000). Such responses were later confirmed and extended by using more extensive arrays and pathogens (Boldrick *et al.*, 2002; Detweiler *et al.*, 2001; Eckmann *et al.*, 2000; Nau *et al.*, 2002). Genes that were prominently induced included many cytokines and chemokines, inflammatory modulators, genes involved in chemotaxis, and genes encoding intracellular signal transduction molecules. While the acute host cell response to various pathogens and PAMPs is not identical, there is considerable overlap between different bacterial pathogens and even viral and parasitic organisms (Boldrick *et al.*, 2002; Granucci *et al.*, 2003; Nau *et al.*, 2002; Zhu *et al.*, 1998).

The discovery and characterization of toll-like receptors (TLRs) explains much of this overlap in gene response to diverse pathogens (Medzhitov, 2001). In humans, there are thought to be ten TLRs that recognize diverse PAMPs such as LPS, CpG DNA, peptidoglycan, and other pathogen-specific molecules (Barton and Medzhitov, 2002; Medzhitov, 2001). However, these receptors seem to feed into a common transcriptional response regulated by NF-κB, leading to a programmed innate response to control the pathogen (Schnare *et al.*, 2001). Thus it is not surprising that Gram-negative pathogens induce quite similar and overlapping responses in inflammatory cells, since LPS and peptidoglycan backbone structures are conserved. Indeed, this response is far more dominant than the response to specific virulence factors and is often the only response seen when virulent and avirulent isogenic strains are compared. The use of host arrays to probe these overlapping responses has considerably enhanced our understanding of what the ''innate response'' consists of, although there are many induced genes that play a role in this response that remain uncharacterized. Comprehensive array analysis with a variety of pathogens and PAMPs will establish a conserved set of genes that should collectively represent this innate response. By comparing more divergent pathogens, such as Gram-positive versus Gram-negative, or virus versus parasite, gene sets can be established for very generic innate responses and more-specific responses to families of related pathogens. Such gene sets can be considered a type of ''molecular signature'' of infection and may even have applications in diagnostic evaluation of uncharacterized microbes.

In one seminal array paper investigating the innate immune response to Gram-negative bacteria (Granucci *et al.*, 2001), Granucci and

colleagues reported that as early as 4 h after Gram-negative bacterial infection, a mouse dendritic cell (DC) line expresses IL-2 mRNA and mouse bone marrow–derived DCs (mBMDCs) express both IL-2 mRNA and protein. Interestingly, bacteria-dependent IL-2 production was DC-specific, as macrophages exposed to the same bacterial stimulus did not induce IL-2 upregulation. IL-2 production by DCs may therefore represent an important step in our understanding of the interaction of DCs with T cells, and possibly also with natural killer (NK) and B cells.

In addition to TLRs, there is another family of receptors that have been recently identified and function much like TLRs yet recognize PAMPs such as peptidoglycan inside host cells. These intracellular receptors belong to the NOD family (for nucleotide-binding oligomerization domain) and have proven to be important molecules used by cells to sense and respond appropriately to the presence of intracellular bacteria (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003a,b; Inohara and Nunez, 2003). Probing the transcriptional response downstream of these intracellular bacterial receptors (specifically Nod-1 and Nod-2) will provide valuable information about their contribution to the innate response, as well as define the signalling pathways they utilize to mediate responses to intracellular antigens. In addition, it is probably very likely that there will be multiple innate response pathways that have not been identified yet, and arrays can help to elucidate these pathways.

It is hoped that by judicious yet comprehensive use of arrays, a more complete library of transcriptional innate responses can be established. It should be emphasized that we currently know very few of the molecules that would comprise such a library. Having such a gene set would aid considerably in defining and prioritizing host genes to study further. In addition, establishing such a library would provide the backbone to begin to identify and develop compounds that stimulate the innate response, thereby enhancing the innate immune system. Such compounds should, in theory, provide potential broad-spectrum therapeutics to a variety of infectious agents, as well as serve as immune modulators for inflammatory diseases. Hints that such compounds might exist come from work done with cationic peptides. Some of these molecules provide considerable overlap in innate responses when added to macrophages compared with that seen when such cells are infected with *Salmonella* (Scott *et al.*, 2000).

The field of innate immune transcriptional responses is in its infancy. However, arrays will rapidly advance this field, and, coupled with appropriate biological experiments, should provide a much more

extensive knowledge about how pathogens respond to host cells, and guide us in identifying compounds that can enhance or alter such responses.

III. Bacterial Arrays

Appreciating the notion that microbial virulence results from a dynamic host-adapted transcriptional response of infecting bacteria, microarrays to monitor the transcriptional repertoire of bacteria in the presence or absence of host cells have been developed and widely used. Microbial genome databases including those at GenBank (www.ncbi.nlm.nih.gov), The Institute for Genomic Research (TIGR; www.tigr.org) and the Wellcome Trust Sanger Institute (www.sanger.ac.uk) are replete with fully sequenced genomes from bacteria with clinical and environmental importance. Many still are in various stages of completion from initial shotgun sequencing to assembly of sequenced contigs and annotation. Many bacterial arrays are now commercially available, including those for *Bacillus subtilis, Helicobacter pylori, Neisseria meningitidis, Streptococcus pneumoniae, Escherichia coli, Salmonella enterica*, and *Pseudomonas aeruginosa*. Several uses of bacterial microarrays can be envisaged and indeed have been cleverly used in the recent literature. These include the use of microarrays for transcriptional profiling of bacterial RNA in a perturbed microenvironment in pure broth cultures or in infection models that take into account the contribution of the host milieu on bacterial adaptation. Bacterial expression profiling presents a special research challenge, and considerations for this experimental design have been well articulated in the recent literature (Conway *et al.*, 2003; Relman, 2002) and will be discussed briefly below. Yet another use involves DNA microarray technology to compare the genetic elements of various unsequenced strains to a sequenced reference strain represented in an ordered array format (Schoolnik, 2002). These experimental schemes will be discussed below and are depicted in Fig. 2 (see color insert).

A. Comparative Genomics

The availability of complete bacterial genomes has made it possible to systematically and comprehensively search lab and clinical isolates of bacteria for conserved and unique gene sequences that may be involved in pathogenesis. The application of microarray technology to genome composition studies—or comparative genomics—has been extremely useful in that it obviates the need for whole genomic

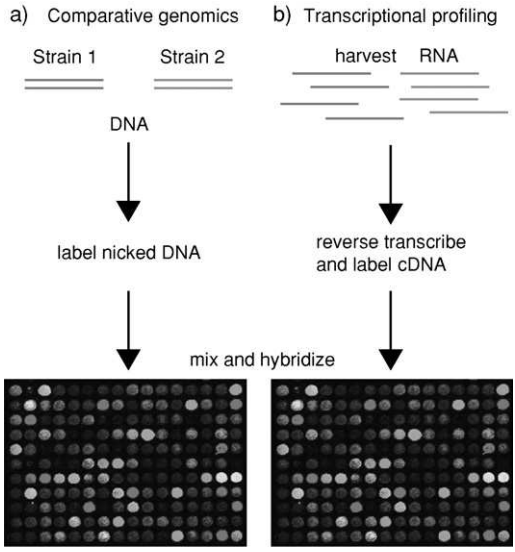

FIG. 2. Microarray-based experimental strategies for studying bacterial gene content and expression. *A*, Comparative genomics compares the genomic content of two different yet related bacterial strains. Genomic DNA isolated from two sources is differentially labelled, mixed, and hybridized to a DNA array that contains complementary DNA fragments from a sequenced reference strain. The level of hybridization intensity for each spot reflects the degree of homology to the complementary DNA represented on the array. *B*, Transcriptional expression analysis of bacterial RNA isolated from two bacterial populations exposed to differential stimuli. Bacterial RNA can be isolated from bacteria growing in pure cultures or from bacteria following infection of cell cultures or *in vivo* tissues. RNA is reverse-transcribed into cDNA and labelled with fluorophores, then mixed and hybridized to the array.

sequencing of multiple microbial strains (Joyce *et al.*, 2002; Schoolnik, 2002). This has allowed a rapid development in our awareness of strain diversity, global gene diversity, and gene synteny, which can then be used to predict how the presence or absence of certain microbial gene products and their interactions with host structures ultimately manifest as disease.

Genome-composition analysis with microarrays involves classifying genes as either conserved or divergent based on the level of hybridization signal on the array. Genes that are present in the genome of pathogenic bacteria but absent from closely related genomes of free-living bacteria or commensals are likely implicated in the unique properties that characterize bacterial pathogenesis. Strain variation can also be determined in this manner whereby clinical bacterial

isolates, for example, can be compared by using a genomic microarray to environmental or laboratory isolates of the same species. Chlamydial genomics, in particular, provides an interesting example of this, showing that *C. pneumoniae* contains 214 open reading frames with no identifiable homologs in the closely related *C. trachomatis* genome or other Gram-negative bacteria (Kalman *et al.*, 1999). Species-specific genes within a common genus may be involved in unique virulence properties that characterize the species, such as host cell tropism, tissue tropism, persistency, and latency. An interesting example of this was reported recently by Perrin and colleagues, who used a *Neisseria* DNA microarray to investigate the genetic basis of *Neisseria* disease pathogenesis (Perrin *et al.*, 2002). *Neisseriae* contribute to a wide spectrum of disease, ranging from meningitis (*N. meningitides*) to genitourinary tract infection (*N. gonorrhoeae*) or are commensal in the nasopharynx (*N. lactamica*). In this study, genetic regions were identified that were either specific to *N. meningitidis* or shared with *N. gonorrhoeae* but absent from commensal *Neisseria*, thus identifying virulence-associated regions for further investigation. The ability of certain obligate intracellular bacterial pathogens to contribute to chronic disease may also be predicated on unique genes giving rise to specialized mechanisms of intracellular survival and the ability to persist in infecting host cells. Genome composition studies using microarrays may be instructive in identifying such gene structures in these bacterial populations that are difficult to work with in cell culture.

Antithetically, genes that are conspicuously absent from a genome can offer as much information about the environmental requirements of growth and pathogenesis as those that are present. In this way, examining what is missing from a bacterial genome can be as instructive as what is present, offering important predictions about how a particular bacteria functions as a pathogen. This was nicely exemplified in a comparative genomics study (Cole *et al.*, 2001) between *Mycobacterium leprae* and *M. tuberculosis*, which revealed that more than 25% of the *M. lepreae* genes (1116 in total) are pseudogenes, containing inactivating mutations, which have collectively eliminated an important repertoire of metabolic activities including siderophore production, elements of the oxidative respiratory chain, and catabolic systems. Two other microarray-based genomic comparisons of *Salmonella* serovars revealed variability in virulence loci which could be clustered into molecular signatures that might define the pathogenic potential and degree of host adaptation of a particular serovar (Chan *et al.*, 2003; Porwollik *et al.*, 2002). Understanding these important

genomic differences between related pathogens by way of microarray-based functional and comparative genomics studies opens the door to assessing the degree to which the expression of unique bacterial genes is reflected at the level of host response and disease manifestation during infection. To begin to explore this concept, Salama and colleagues (Salama *et al.*, 2000) used a whole-genome microarray representing two sequenced strains of *Helicobacter pylori* to examine the genetic diversity of 15 different strains. This particular study identified a common ''core'' set of genes present in all the strains tested but also revealed 362 open reading frames (representing 22% of the genome) that were absent from one or more of the tested strains. In a follow-up study using the same *H. pylori* array, Israel and colleagues (Israel *et al.*, 2001) identified two clinical isolates of *H. pylori* that differed in their pathological outcomes in a rodent model of *H. pylori* infection—one being able to cause more severe gastritis, proliferation, and apoptosis than another strain, which could not induce gastric ulceration and atrophy. The microarray data demonstrated that the less-pathogenic strain had incurred a large deletion in the *cag* pathogenicity island that encodes a type IV secretion system, thereby identifying bacterial determinants directly related to the pathogenic potential of the organism. In contrast, an examination of the genomic content of 18 clinical and environmental isolates of *Pseudomonas aeruginosa* (Wolfgang *et al.*, 2003) revealed a striking conservation and synteny of genome content that could not be immediately correlated with the strain's respective abilities to infect immunocompromised patients and individuals with cystic fibrosis. In aggregate, the above studies have demonstrated the power of microarray-based comparative genomics studies to uncover conserved genetic elements related to core functions and pathogenesis and also how variable disease outcomes can ultimately be traced back to unique bacterial virulence determinants present in the genome.

Recently, novel statistical algorithms designed specifically for assessing microarray-based comparative genomics studies have been developed (Kim *et al.*, 2002). These methods were developed to help overcome the inherent subjectivity in setting array cut-off values for present and absent genes, which might lead to misclassification of gene content. Indeed, the methods used by Kim and colleagues lead to the reassignment of hundreds of genes from previous comparative genomics microarray studies of *Helicobacter pylori* and *Campylobacter jejuni* strains (Kim *et al.*, 2002). Yet the challenges arising from comparative microarrays to study genetic diversity and virulence propensity of related microorganisms remain. While these studies provide one

with a list of conserved and divergent genes, the relevance of these studies is realized only when the phenotypic differences that are observed between strains can be correlated with the genotypic differences that are defined by the array data.

B. Transcriptional Profiling in a Perturbed Bacterial Microenvironment

An important application of microarray technology as it relates to microbial pathogenesis is transcriptional profiling of microbial genes under controlled *in vitro* conditions that are intended to simulate a specific stage or environment encountered during host interaction. Of particular interest are ongoing studies investigating the coordinated expression of virulence factors required for bacterial pathogenesis. An understanding of regulated bacterial gene expression during exposure to various environmental cues encountered in a host environment can provide new functional assignments for putative virulence factors due to the high degree of temporal co-regulation of bacterial genes required for common functions (such as pathogenesis). Not surprisingly, these types of studies have confirmed that most bacteria are parsimonious when it comes to gene expression, exhibiting tightly regulated gene expression profiles and making gene products only when they are necessary during growth and survival. This is exemplified in a recent array study looking at global analysis of RNA decay, where 80% of *E. coli* transcripts had a half-life between 3 and 8 minutes, with some transcripts decaying on the order of seconds (Bernstein *et al.*, 2002). It is these properties of bacterial gene regulation and RNA turnover that makes bacterial transcriptional profiling technically demanding and inherently difficult. With a few very recent exceptions (Belland *et al.*, 2003; Eriksson *et al.*, 2003; Schnappinger *et al.*, 2003; Staudinger *et al.*, 2002) (discussed in Section II.C), bacterial transcriptional profiling has been limited to bacteria grown under *in vitro* conditions that are meant to imitate various defined stages of infection, such as iron and nutrient limitation (Baichoo *et al.*, 2002; Guedon *et al.*, 2003), the acidic environment of the macrophage phagosome (Fisher *et al.*, 2002) or the gut (Merrell *et al.*, 2003), anaerobic conditions thought to be involved in some bacterial diseases (Sherman *et al.*, 2001; Ye *et al.*, 2000), and immune insults from cationic antimicrobial peptides (Bader *et al.*, 2003). While these types of *in vitro* studies have been imposed because of technical constraints limiting the reliable and quantitative recovery of intact bacterial RNA from complicated samples, they are nonetheless germane to the infectious process, providing molecular

insight into biological features of persistence and pathogenesis. For example, in *Mycobacterium tuberculosis* infections, the basis for bacterial persistence within host tissues has been linked to hypoxic conditions encountered within fibrous granulomas in the lung (Yuan *et al.*, 1998; Zahrt, 2003; Zahrt and Deretic, 2001), which promote bacterial entry into a clinically latent state. When using an *M. tuberculosis* whole genome microarray following exposure of bacteria to low oxygen tension, more than 100 genes were identified whose expression was regulated in a hypoxic state (Sherman *et al.*, 2001). While the precise role of these genes in the establishment of the latent state in tuberculosis disease remains unresolved, the ancillary value of these initial experiments was the identification and subsequent characterization of a two-component response-regulator transcription factor that mediates the hypoxic response of *M. tuberculosis* (Park *et al.*, 2003).

In a recent study involving *Helicobacter pylori* (Thompson *et al.*, 2003) the global pattern of growth-phase-dependent gene expression was monitored in a comprehensive time course using a 1660-element array representing each open reading frame in two sequenced strains of *H. pylori*. Following cluster analysis of the expressed genes, a major transcriptional switch was identified between the late log to stationary phase transition, which involved many genes related to virulence as well as unknown co-regulated genes. This study provides an excellent example of how microarrays can help assign function to unknown genes based on their co-regulation and temporal appearance with known virulence-associated genes. While the importance of these unknown genes remains inferred, it does provide clues and a legitimate rationale for further study with more traditional hypothesis-driven approaches in defined biological systems. Such approaches should sort out whether virulence gene expression during transcriptional phase shift is ultimately related to the virulence of this bacterium during a natural infection. Collectively, these studies highlight that transcriptional profiling of bacterial gene expression under defined *in vitro* conditions can serve as a practical and testable surrogate of expression patterns during various stages of infection.

Firoved and Deretic discuss the use of bacterial mutant strains to discover genes that are transcriptionally co-regulated with previously-described virulence factors (Firoved and Deretic, 2003). The emergence of mucoid *P. aeruginosa* in cystic fibrosis patients is associated with respiratory decline and poor prognosis. The conversion to mucoidy is usually caused by mutations in the *mucA* gene, resulting in activation of AlgU. The authors used microarrays to find other genes that are co-induced with AlgU. Brazma and Vilo discuss in greater detail the

use of microarrays in the discovery of regulatory signals via the analysis of conserved sequences in the promoters of genes that cluster together in a common transcriptional response (Brazma and Vilo, 2000).

Several groups have used bacterial microarrays to determine the effect of antibiotic treatments or to find antibiotic susceptibility factors. Mongodin and co-workers studied the differences between *S. aureus* strains with intermediate and high resistance to vancomycin (Mongodin *et al.*, 2003). They found that the major difference conferring increased resistance is in the purine biosynthetic operon. In an early microarray study, Wilson *et al.* examined the changes in *M. tuberculosis* gene transcription as a function of treatment with isoniazid (Wilson *et al.*, 1999). It should be noted that conclusions from this type of study could be confounded by potential differential metabolism and penetration of the antibiotic under *in vivo* conditions compared to that *in vitro*. These studies have provided important information about the transcriptional response of bacteria in pure cultures following exposure to defined environmental cues. Important studies are now emerging that aim to profile the transcriptional response of bacteria within the context of a host cell or host tissue background.

C. Bacterial Arrays Within a Host Milieu

The fastidious growth requirements of intracellular bacteria and the increased complexity of the host background present a special research challenge when performing bacterial microarray experiments from infected host material. Nevertheless, progress into the interactions that occur between bacteria and host cells, including the molecular mechanisms of host-bacteria interactions, has been made possible by employing well-defined cell culture models of infection together with novel cell and animal models. As such, expression profiling within intact hosts has been largely limited to cell culture models of infection, with a few recent exceptions that will be discussed in Section IV. The use of microarrays to survey the entire spectrum of bacterial gene expression following infection may allow one to (i) predict the function of unknown genes based on their temporal appearance, (ii) characterize the transcriptional response to environmental cues or pharmacologic inhibitors, (iii) define a global gene signature of infections, and (iv) correlate the expression of putative virulence-associated genes with a given pathology.

As mentioned, species-specific genes within a common bacterial genus may be involved in unique virulence properties that characterize

the species such as host cell tropism. One application of a bacterial array is to identify a unique bacterial gene set from a single species that is required for colonizing various cell types. This type of bacterial array approach has proven challenging from a technical perspective, given the inherent complexity of the RNA population under study (Conway and Schoolnik, 2003). The lability and tightly regulated nature of bacterial RNA often necessitates optimized procedures for quantitative recovery and enrichment of bacterial RNA. Dietrich and colleagues (Dietrich *et al.*, 2003) used this type of microarray approach to identify *Neisseria meningitidis* genes that were involved in host cell tropism. *N. meningitidis*, a causative agent of septicemia and meningococcal meningitis, is a unique pathogen in that its natural course of infection requires colonization of distinct cell types at various stages of infection. Initial colonization of the nasopharyngeal epithelial lining is followed by systemic infection, hematogenous dissemination, and infection of endothelial cells lining the blood-brain barrier. Using a 2,158-element oligonucleotide array representing each open reading frame in *N. meningitidis*, Dietrich and colleagues found a subset of virulence genes required for colonization of both epithelial and endothelial cells. Interestingly, a unique subset of bacterial genes was identified that were involved only in colonization of either epithelial cells or endothelial cells. The identification of cell-type specific bacterial genes underscore the notion that invading bacteria encounter a dynamic host environment for which they must constantly adapt to successfully colonize the host. These data also insinuate that bacteria are able to sense this dynamic and specific intracellular environment and adapt their transcriptional program accordingly. These types of array studies will be important for pathogenic bacteria whose virulence program requires infection of multiple cell types during infection, such as *Salmonella* infection of epithelial cells, M-cells and macrophages, or the infection by *Chlamydia pneumoniae* of lung epithelial cells, alveolar macrophages, and endothelial cells.

In contrast to facultative intracellular bacterial pathogens that can be processed for microarray experimentation from either pure broth cultures or from infected cell material, obligate intracellular bacteria dictate the type of viable experimental design. One obligate intracellular organism that has been studied in this context is *Chlamydia trachomatis*, a causative agent of endemic blinding trachoma, lymphogranuloma venereum, urethritis, ectopic pregnancy, and tubal infertility in women. With a parasitic lifestyle and no cell-free growth system, the host cell is the only machine capable of replicating this bacterium. The fastidious growth requirement of obligate intracellular bacteria is

further hampered by the lack of a genetic transformation system, thus precluding our ability to generate genetic mutants. Genome sequencing has arguably impacted chlamydial research more positively than for other Gram-negative bacteria because of these limitations imposed by the growth characteristics of the organism. Using microarrays to profile a highly choreographed and sophisticated developmental cycle such as *Chlamydia* seems like a reasonable approach to help overcome at least some of these limitations, and indeed, these technical challenges appear to have been overcome with the publication of two independent reports describing transcriptional profiling of the *C. trachomatis* developmental cycle (Belland *et al.*, 2003; Nicholson *et al.*, 2003). These studies are valuable for several reasons, not the least of which is that they help define the mechanistic elements and temporal events involved in production of a viable infectious organism. The ancillary value of these studies is the development of practical methods for bacterial RNA isolation from infected cell material, removal of host cell messenger RNA and specific degradation of contaminating ribosomal RNA from both the host and bacteria. At least in the work by Belland and co-workers (Belland *et al.*, 2003), these steps were necessary for reproducible and consistent array results.

Bacterial array studies in the context of a host cell background are highly relevant for the study of host-pathogen interactions. We now appreciate the fact that host genotype can have a profound influence on the expression of microbial virulence genes (Bellamy, 2003; Casadevall and Pirofski, 2001; Zaharik *et al.*, 2002), whereby the presence of innate host resistance loci and innate immune activation factors can modify virulence gene expression in the bacteria. A recent seminal paper (Schnappinger *et al.*, 2003) provides an excellent example of how the host genotype and the presence of activators of innate immune function induce an adaptive transcriptional program in bacteria to counteract host defences. Schnappinger and colleagues (2003) used a *Mycobacterium tuberculosis* microarray to determine the transcriptional responses of intraphagosomal *M. tuberculosis* residing in macrophages from either wild-type or nitric oxide synthase (NOS) 2-deficient mice before and after macrophage activation with interferon gamma (IFN-γ). Interestingly, intraphagosomal *M. tuberculosis* responded differentially to naïve macrophages or macrophages stimulated with IFN-γ following infection, which was found to be dependent on the production of nitric oxide from wild-type mouse macrophages since the IFN-γ-specific gene set was not induced in bacteria residing in phagosomes from $NOS2^{-/-}$ mice. These data suggest that intracellular pathogens residing in specialized niches such

as modified phagosomes can uniquely adapt to not only the luminal contents of these vacuoles to induce the requisite metabolic adaptations but also modify their transcriptome in such a way to resist killing by innate immune host defenses. This notion is supported by other recent array studies of intraphagosomal *E. coli* ingested by wild-type and phagocyte-oxidase-deficient human neutrophils (Staudinger *et al.*, 2002) and of intravacuolar *Salmonella enterica* serovar Typhimurium residing in murine macrophages (Eriksson *et al.*, 2003). In this latter study, the global profile of *Salmonella* gene expression was determined from bacteria inside *Salmonella*-containing vacuoles in macrophages. These data indicated that the intravacuolar environment encountered by *Salmonella* is not limiting for iron or amino acids and that the vacuole is low in phosphate and magnesium but high in potassium. Clues as to the sugars used as a carbon source for intracellular bacteria was inferred from the array data, but interestingly, almost half of the intracellular-regulated genes were of unknown function, suggesting that intracellular growth requires novel macrophage-associated functions. Bacterial microarrays offer the promise of uncovering even more examples of how the host genotype (the presence or absence of certain loci, or even single gene polymorphisms), tailors the bacterial response during infection and how this adaptive response of the bacteria manifests in disease.

IV. Towards *In Vivo* Tissue Sampling

Obtaining expression profiles from a microorganism within a host cell milieu in tissue remains the ''holy grail'' of microarray technology as it applies to microbiology and infectious diseases. But technical hurdles in obtaining enough high-quality bacterial RNA from a complex background of host cells cannot be overstated. This is true because of (i) the often low bacterial loads encountered in infected tissues, (ii) heterogeneous distribution of pathogens within the tissue, (iii) the presence of other commensal bacteria in the samples, (iv) the ever-vexing RNA degradation issues, and (v) tissue sampling error, which arises because infected tissues are sometimes not macroscopically identifiable. However, these technical challenges are now being overcome, and studies using microarray technology to transcriptionally profile *in vivo*-grown bacteria are emerging, often using very unique infection models. For example, DNA microarrays have been ingeniously used to survey the adaptive genetic responses of *Borrelia burgdorferi*, the causative agent of Lyme disease, to growth in dialysis membrane chambers implanted in the peritoneal cavity of rats (Revel *et al.*, 2002).

In a seminal paper (Merrell *et al.*, 2002), Merrell and colleagues studied the epidemic spread of *Vibrio cholerae* from naturally occurring infections. Cholera is an endemic waterborne diarrheal disease in much of the developing world including Asia, Africa, and Latin America and kills 50 to 70% of untreated patients (Faruque *et al.*, 1998) making it a major public heath problem. *V. cholerae* shed from the human gastrointestinal tract exhibited greatly enhanced infectivity compared to laboratory-passaged bacteria following mixed oral inoculation of mice—a phenotype that was entirely lost if stool-isolated bacteria were cultured in broth but retained if bacteria were incubated in pond water prior to inoculation—suggestive that passage through the human intestinal tract promotes and maintains infectivity in a natural aquatic environment. Cholera bacteria isolated from stools were subjected to a *Vibrio* microarray, which identified 44 genes that were induced and 193 genes that were repressed in human-shed *V. cholerae* when compared to laboratory-grown bacteria prepared in a similar fashion to that used in the mouse competitive infection experiments. These data beg the question of whether other bacteria responsible for epidemic infections use a similar host-induced mechanism for enhanced infectivity. This first use of microarray data to shed light on potential targets to interrupt epidemic transmission of pathogenic bacteria has enormous implications for improving public health.

In a second influential paper by Xu and colleagues (Xu *et al.*, 2003), the transcriptome of *V. cholerae* was determined in rabbit ileal loops, a model infection of the small intestine in which bacterial replication and pathogenesis would occur during a natural human infection. The genomic transcriptional pattern of bacteria grown in the small intestine was then compared with laboratory-grown *V. cholerae* under aerobic conditions to identify those genes expressed specifically in the small-intestine environment. Prominent findings included the preferential *in vivo* induction of 24 genes involved in iron transport and storage and 13 anaerobic energy metabolic genes, suggesting that *V. cholerae* encounters an iron-limiting, anaerobic, and nutrient limiting environment in the small intestine. Perhaps more interesting was the significantly enhanced *in vivo* expression of virulence factors including those involved in adherence, motility and regulation of cholera toxin and the toxin-coregulated pilus. This finding insinuates that *V. cholerae* senses and responds specifically to its intestinal environment to activate a virulence program leading to the characteristic fluid loss seen during cholera infection and epidemic spread of the organism (Faruque *et al.*, 1998).

Boyce and colleagues have studied the transcriptional state of *Pasteurella multocida*, the causative agent of fowl cholera during infections of the natural chicken host (Boyce *et al.*, 2002). Although technical challenges limited this study to only three chickens, they concluded that a clear difference in bacterial gene expression was observed from each infected chicken, suggesting that individual host variation is extremely important to pathogen gene expression. Further, the Gordon laboratory has contributed two important microarray studies aimed at *in vivo* analysis of host-pathogen interactions. Hooper *et al.* examined the influence of commensal bacteria by studying the impact of *Bacteroides thetaiotaomicron* on intestinal gene expression. They used a germ-free mouse system and compared mice colonized by *B. thetaiotaomicron* for 10 days to germ-free mice (Hooper *et al.*, 2001). These studies allowed the discovery of microbicidal activity of the angiogenin gene family (Hooper *et al.*, 2003). Last, Mysorekar *et al.* used adult female mice to explore the host factors responsible for regulation of uroepithelial renewal and host defense during infection by uropathogenic *Escherichia coli* (UPEC) (Mysorekar *et al.*, 2002). They inoculated mouse bladders via transurethral catheter and analyzed host RNA after several hours. The results stressed the importance of the FimH adhesion to pathogenesis.

A common theme in at least some of these *in vivo* microarray infection studies is that the *in vivo* sample used for isolation of bacterial RNA was relatively acellular with respect to host cells and commensal bacteria (dialysis chamber implants and stool). As discussed, this can aid in reproducibility and reliability of microarray data from *in vivo* samples because of the lack of contaminating host material. Yet this same fact may also introduce an acceptable yet noteworthy caveat in that *in vivo* grown bacteria are taken out of context of host cell contact, which might very likely be required for pathogenesis during natural infections. The aforementioned caveats and technical pitfalls notwithstanding, these *in vivo* studies of bacterial gene expression are categorically groundbreaking and will likely reveal colonization and transmission stratagems in response to a cognate pattern of host defense that are responsible for disease-causing phenotypes.

V. Technical Aspects of the Host-Pathogen Interaction

A. Platforms and Detection Schemes

An inherent limiting factor in the utility of microarrays is the ability to detect low abundance transcripts. Recently a superior labelling scheme has been developed that may allow the use of reduced amounts

of RNA, thereby facilitating studies of rare mRNA populations. Most microarray experiments to date have utilized fluorescent labelling (Cy3/5) of cDNA during reverse-transcription of harvested RNA. These applications often require ~10 μg of RNA per slide. Recently a technology has been developed that allows the use of one tenth the standard amount of RNA relative to Cy3/5 fluorescent technology. This new technology utilizes the principles of resonance light scattering (RLS), in which microscopic gold and silver particles scatter white light, with the resultant signal behaving as a fluorescent analog (Bao *et al.*, 2002). Biotinylated or fluorescein-labelled cDNAs are generated by reverse transcription and hybridized to microarrays in a similar way to that described for Cy3/5 dye detection. After washing, the slide is incubated at room temperature with a solution containing 80-nm diameter gold particles coated with an anti-hapten antibody. The gold particles bind specifically to the hybridized biotinylated targets and excess particles are washed away. The slide is then scanned by using a specially designed white light CCD scanner. Silver particles are also available for use in two-color assays, as depicted in Fig. 3 (see color insert). We have employed this technology in our studies of the host response to EHEC and EPEC infection and have observed superior signal-to-noise ratios when using RLS technology with only 2 μg of RNA, relative to 10 μg input RNA with Cy3/5 technology.

B. Sampling Bacterial and Host RNA

As described above, the basic microarray framework as it relates to host-pathogen interactions consists of an ex vivo measurement of gene expression in host cells before and after infection with a pathogen (Kato-Maeda *et al.*, 2001). We have also described applications in which microarrays are utilized to study genetic polymorphism, co-regulated virulence genes (Eckmann *et al.*, 2000), and new drug targets (Marton *et al.*, 1998; Wilson *et al.*, 1999). However, there are numerous confounding aspects (both biological and technical) that one must consider to conduct a statistically sound and biologically relevant microarray experiment. A number of these issues will be discussed below. Experimental design issues relating to enhancing precision and accuracy of microarray measurements have been discussed in detail (Yang and Speed, 2002; Yang *et al.*, 2002), while Churchill discusses methods to increase statistical power in two-colour cDNA microarrays (Churchill, 2002).

When performing infection-related expression studies, several biological factors must be taken into account. For example, studies

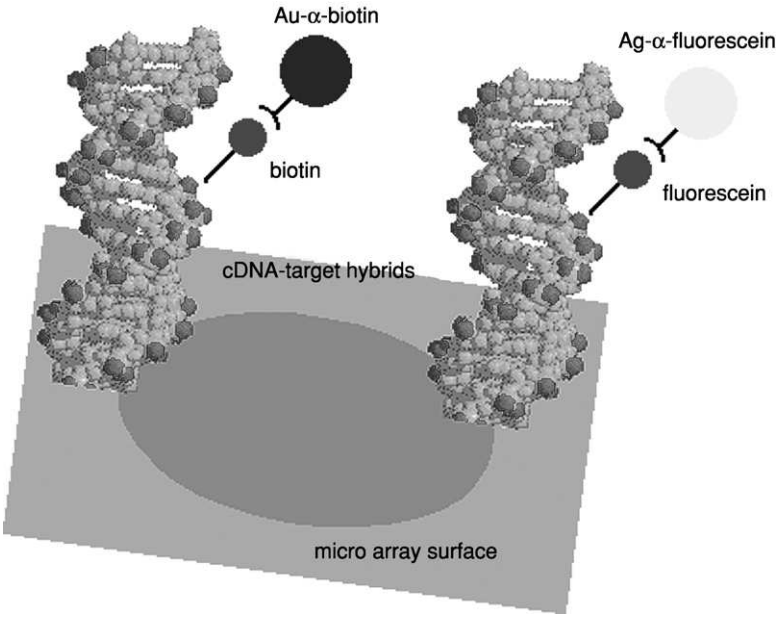

FIG. 3. Resonance Light Scattering detection of microarray hybridization. The GeniconRLS™ technology is based on nanometer-sized gold or silver particles that generate intense, monochromatic scattered light signals on white light illumination. Biotin- or fluorescein-dUTPs incorporated during cDNA synthesis are detected by using gold or silver particles conjugated to anti-hapten antibodies after the target cDNA is hybridized to DNA probes immobilized on a glass slide. Following illumination with white light, metal particles scatter light with an intensity that is proportional to the number of hybridized particles on the array. The array itself can also be archived and used for future scanning, detection, and imaging.

attempting to identify effector protein function through the use of host transcriptional profiling are best accomplished by using different strains and restricted mutants of a bacterial species derived from clinically relevant, isogenic wild-type strains. Yet because different strains often display inherent differences in infectious dose and growth rates following infection of a host cell, one must be cognisant of these potential differences as they may lead to differential changes in the host environment that is independent of any pathological mechanism. These types of considerations are imperative if scientifically- and clinically meaningful data are to be generated (Maiden and Feavers, 2000).

Especially difficult are comparisons among Gram-negative bacterial mutants lacking functional type III secretion systems. Such strains can differ quite strongly in their ability to adhere to host cells, as well as in

the kinetics of host cell interaction. One approach to help address this issue is to use time-course studies to help determine whether a datapoint obtained at a single time point may be compromised by small phase shifts in the kinetics of host response (Diehn and Relman, 2001). In such systems using wild-type and mutant bacteria, or when comparing the host response to different bacterial species, it is sometimes unfeasible to add equal colony-forming units and expect to achieve similar infectious dosage. Ideally, one can normalize several infection parameters prior to an array experiment to help overcome this technical constraint. To this end, we have sought a method to compare the host response to EHEC and EPEC at early time points of infection. Although these bacteria infect host cells in a similar manner, they differ greatly in adhesion, growth rate, and kinetics of protein delivery. We therefore normalized the infections by monitoring the kinetics and magnitude of delivery of the Tir effector protein into host cells. Tir, or translocated intimin receptor, is delivered to the host cell plasma membrane in a type III secretion-dependent manner and can be monitored by using immunofluorescence. Such approaches allow greater confidence that the infections between organisms are as experimentally similar as possible and minimize the number of ''false-positives'' that would otherwise result from confounding differences in the host cell environment. Failure to control for such biological differences limits the ability to compare genomic datasets from various sources (Diehn and Relman, 2001). Despite these caveats, if carefully controlled and well-designed, microarray studies using virulence factor mutants and isogenic wild-type strains will provide much new information about the molecular and cellular basis of bacterial pathogenesis and begin to explore an important and exciting new area of pathogenesis research for which little is currently known—delineating how the unique interactions between host components and bacterial virulence factors manifest in disease.

Equally important is the development of a standardized methodology of reporting microarray data to facilitate interlaboratory comparisons of datasets. The reader is invited to consult Stoeckert *et al.* for a discussion of the standards and methods to report experimental design to facilitate comparison among different microarray datasets (Stoeckert *et al.*, 2002). Brazma and co-workers have proposed the minimum standards for a useful reporting of microarray data and infection conditions, called Minimum Information About a Microarray Experiment (MIAME) (Brazma *et al.*, 2001), which our laboratory has implemented.

An additional conceptual consideration is how relevant the measurement of an acute epithelial cell response is to the biology of a more

chronic infection. We have discussed above the limitations of chlamydial studies that focused on the host response to acute infections and the need for initiatives to monitor bacterial gene expression during persistent infections. These types of studies are becoming more realistic with the development of cell culture models of persistent bacterial infections and animal models that are able to support chronic bacterial infections. The combination of laser capture microdissection technology to capture infected cells *in vivo* may allow the study of longer timecourses of infections—on the order of days, rather than hours—with various pathogens.

Conway and Schoolnik review experimental difficulties inherent to microarray experiments to study bacterial gene expression (Conway *et al.*, 2003). In most systems, the limited number of microorganisms and the presence of host cells and normal microflora can preclude *in vivo* experimentation in the absence of technological improvements in RNA amplification and the reproducible and quantitative separation of host and bacterial RNA populations. It is therefore common to use *in vitro* conditions intended to simulate host microenvironments that an infectious organism might encounter. Even in carefully controlled systems, simple variation in transcription initiation frequency and mRNA turnover confounds many bacterial array experiments. Several authors have commented on the phenomenon that interconnection between regulatory networks may result in the observance of seemingly unrelated genes responding to similar experimental variables (Conway *et al.*, 2003; Watts and Strogatz, 1998).

When attempting to compare different gene expression datasets, important differences in pre-array methodology must be considered, such as differences in RNA handling and hybridization, which may affect direct inter- and intra-laboratory comparisons of array datasets (Diehn and Relman, 2001). It is also important to consider whether different expressed sequence tags representing the same gene may yield dissimilar hybridization intensities, whether the method of priming cDNA synthesis can influence the distribution of molecules, and whether hybridization with cDNA generated from total RNA versus mRNA is also an important variable (Getz *et al.*, 2000). On the other hand, it has been suggested that perhaps little significance should be ascribed to large differences in the magnitudes of array data points, as microarrays tend to behave more semi-quantitatively and do not always directly correlate with quantitative reverse transcription (RT)-PCR results (Vasil, 2003). Hooper and colleagues highlighted a potential difficulty in cross-hybridization of related gene products (Hooper *et al.*, 2003). They used a GeneChip probe designed to detect *ang3*

mRNA transcripts coding for angiogenin-3 in a study of the influence of commensal bacteria on intestinal gene expression. However, on sequencing of a cDNA clone derived from the transcript, they observed that a related transcript, *ang4* (81% identity), was responsible for hybridization to the microarray. While this careful observation allowed the fortuitous discovery of microbicidal family of angiogenins (Hooper *et al.*, 2003), it also underscored the fact that array hybridization signals can be influenced by related paralogs in a genome.

VI. Data Validation Strategies

A. *In Vitro* Validation

Perhaps one of the most challenging aspects of microarray analysis of host-pathogen interactions is independent validation of the results, both at the level of confirming RNA quantitation and in verifying the biological relevance of such findings. Chuaqui and co-workers have recently highlighted the primary issues and methodology available for confirmatory assays (Chuaqui *et al.*, 2002). After surviving numerous statistical criteria, a subset of transcripts showing differential expression among experimental conditions is generally examined with semi-quantitative RT-PCR or Northern blotting. General agreement with the microarray data allows greater confidence in the data. However, Conway and Schoolnik point out that a direct comparison to RT-PCR shows that microarrays may underestimate induction ratios by 2–10 fold (Conway *et al.*, 2003). Transcripts of greater interest are often further explored with quantitative RT-PCR, although start-up costs of this technology are high and there are concerns about the ability to validate genes that are not highly differentially regulated.

After the first-pass confirmation of gene expression levels, the data are then often considered in the context of previously published experiments to ascertain whether the observed results make sense in a global biological setting. For example, the observation of up-regulation of cytokines and proinflammatory cell signalling pathways in the context of a host response to an enteric bacterium would not be unexpected. Comparisons between different array datasets will become more constructive when there exists a standardized reporting of microarray data (Chuaqui *et al.*, 2002).

Ideally, one is able to validate gene expression ratios in an *in vivo* setting. In this regard, de Grado *et al.* nicely validated EPEC-mediated up-regulation of *egr-1* by isolating total RNA from infected mice (de Grado *et al.*, 2001). An additional consideration for data validation

is defining the bacterial component(s) responsible for an observed host response by following up large arrays with quantitative RT-PCR following infection with well-defined and restricted bacterial mutants. Complementary experiments can be designed to examine gene expression in specific cell types (Hooper and Gordon, 2001), including cells neighboring to infected cells, as these cell types may also have important roles in pathogenesis (Vallance *et al.*, 2002). This is one area in which laser capture dissection microscopy of specific cell populations could have a direct application.

After confirmation of transcript levels, expression of the corresponding protein is often evaluated either by Western blotting or by using immunohistochemistry (Chuaqui *et al.*, 2002). It is interesting to compare the degree of correlation between protein and mRNA levels in both bacteria and host cells. It has been observed in several bacterial systems that enzyme levels may correlate reasonably well with transcript levels (Arfin *et al.*, 2000; Eynmann *et al.*, 2002; Yoshida *et al.*, 2001). However, in more complex systems, this degree of correlation is unclear (Griffin *et al.*, 2002; Gygi *et al.*, 1999). Indeed, in samples examined with tissue microarrays at the National Cancer Institute, significant correlation between mRNA and protein levels were observed in less than half the cases (Chuaqui *et al.*, 2002). Additionally, the mere presence of a protein does not imply an active protein, as post-translational modifications and other regulatory controls must also be considered.

We have begun to explore this issue by comparing our host microarrays from EPEC infection to a complimentary study of the host proteome during infection under identical conditions. We used isotope-coded affinity tagging (ICAT) to compare the peptide abundance in Caco-2 cells treated with EPEC 2348/69 and EPEC 2348/69Δ*escN*. Peptide expression ratios were then compared with gene expression ratios from infections performed under identical conditions. Figure 4 displays representative data in which mRNA expression ratios are plotted vs. protein expression ratios. We observed that when analyses are restricted to enzymes that are up regulated during wt EPEC infection, there is significant correlation between datasets (Fig. 4A). This correlation is significant for mRNA samples obtained at the same time as protein samples (4 hr; correlation coefficient = 0.82) and for mRNA samples obtained 1 hr prior to protein sampling (3 hr; correlation coefficient = 0.88). Greater variability between mRNA and protein expression is observed in datasets comparing the expression of all host enzymatic functions (Fig. 4B) and all upregulated host proteins (Fig. 4C). As organism-wide sampling of mammalian proteomes

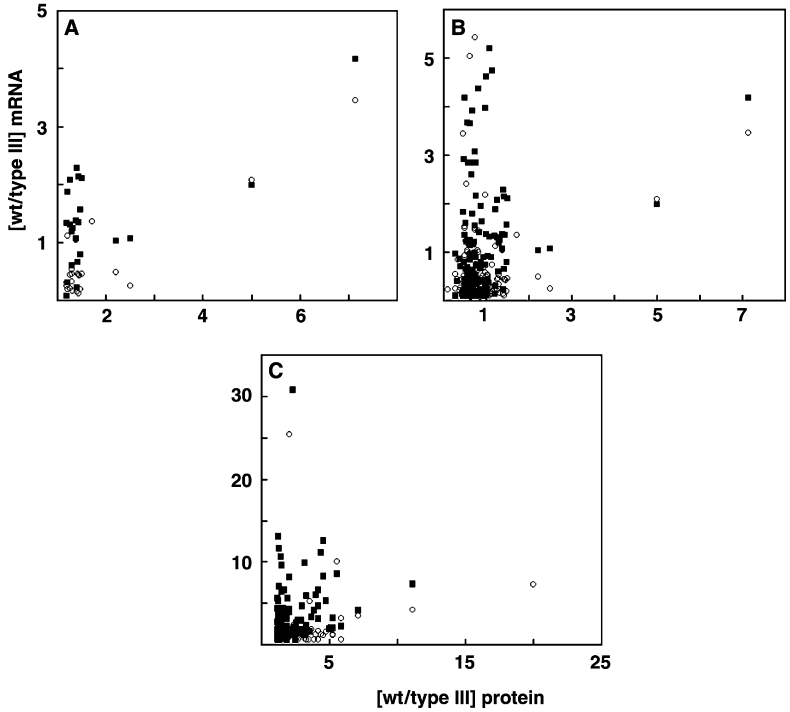

FIG. 4. Relationship between differential mRNA and protein expression in host cells during EPEC infection. *A*, Correlation between mRNA and protein expression ratios in enzymes up regulated in Caco-2 cells by infection with EPEC 2348/69. Data are plotted as the [EPEC2348/69/EPEC 2348/69Δ*escN*] mRNA expression ratio (3 hr post infection, open circles; 4 hr, closed squares) vs. the [EPEC2348/69/EPEC 2348/69Δ*escN*] protein expression ratio (4 hr). N = 24. *B*, All host enzymes identified in proteomic analysis. Symbols as in *A*. Note the differences in the scale of axes. (3 hr, N = 158; 4 hr, N = 144). *C*, All host proteins (including non-enzymatic functions) up regulated in Caco-2 cells by infection with EPEC 2348/69. Symbols as in *A*. Note the differences in the scale of axes (3 hr, N = 160; 4 hr, N = 143).

becomes more commonly implemented, it is likely that careful comparisons in other biological systems will be made.

B. BIOLOGICAL VALIDATION

From a host perspective, even if differences in protein activity can be measured following primary identification in a microarray experiment, it can be difficult to assess the importance of a specific host pathway to pathogenesis. Fibroblast cell lines and knockout mice are available, but

such applications are often limited to studies of the host immune response or other non-essential factors. Gene knockdown with RNA interference (RNAi) is an emerging technology that may facilitate the precise study of mammalian gene function as it relates to interaction with bacterial components and the progression towards a specific disease state (Lieberman *et al.*, 2003) and perhaps be used therapeutically for certain diseases (Song *et al.*, 2003a; Song *et al.*, 2003b). Other investigators have sought to use organisms with tractable genetic systems to study bacterial pathogenesis. Yeast models systems have been creatively employed to study bacterial effector function (Lesser and Miller, 2001), and yeast strains with single gene deletions are available for study of the host contribution to the host-pathogen interaction. *Caenorhabditis elegans* is also being developed as a model organism for pathogenesis studies, especially in the context of genes involved in toxin-mediated killing (Alegado *et al.*, 2003).

VII. Conclusions and Future Prospects

Microarrays offer the promise of working toward a ''systems based'' model of infectious diseases, toward an integrated understanding of pathogenesis that takes into account the adaptive and counter-adaptive strategies of both host and microorganism. Indeed, as evidenced in the recent literature, microarrays are fulfilling this promise. Seminal studies with microarray technology have been made possible through concurrent advances in bioinformatics and statistics that enable data from these studies to be manipulated and accessed in resourceful ways. Further breakthroughs will be facilitated by the ''open-access'' concept applied to microarray data whereby complete datasets are freely available online for comparative studies from several microarray experiments, which will facilitate independent corroboration and authentication of data and ultimately the generation of novel testable hypotheses. Online microarray databases such as the Stanford Microarray Database (http://www.dnachip.org) are leading this front by providing an open resource in which full microarray datasets are released to the public at the researcher's discretion or on publication of the work in peer-reviewed journals.

We have highlighted some recent advances in the applied use of microarrays in bacterial pathogenesis research. Without doubt, these studies have advanced our understanding of bacterial pathogenesis and enlightened us to the intricacies and extensiveness of the host-pathogen interaction. These advances will continue to define a new period in our understanding of pathogenesis whereby both the host

and pathogen interact in a dynamic nature to instigate disease. In fact, this new era is already being ushered in to shape a new host-pathogen paradigm.

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The Inactivation of Microbes by Sunlight: Solar Disinfection as a Water Treatment Process

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I. Introduction

The use of sunlight is one of the oldest recorded methods of water purification, dating back to at least 2000 BC in the Sanscrit text ''Oriscruta Sanhita'' (Patwardhan, 1990). However, it is only in more recent times that the underlying scientific basis of this approach has been established. The first systematic study into the inhibitory effects of solar radiation on bacteria was that of Downes and Blunt (1887), who reported that the development of bacteria in nutrient broth and urine could be halted by exposure to the sun and that such solutions ''may be absolutely and perfectly sterilized by sunlight.'' They further demonstrated that the spores of mycelial fungi are more resistant than bacterial cells to the inhibitory action of sunlight, and they also showed that short-wavelength solar radiation has the greatest antimicrobial effect.

Almost a century later, Acra and co-workers proposed the practical application of sunlight for the disinfection of oral rehydration solutions and drinking water (Acra *et al.*, 1980) in a process usually termed

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solar disinfection (Acra *et al.*, 1984, 1989). They envisaged that sunlight might be able to provide a low-cost, sustainable, and simple method of treating contaminated drinking water in developing countries with consistently sunny climates under circumstances in which people had no access to alternative water treatment systems. At its simplest (batch-process solar disinfection), the method involves filling a transparent glass or plastic vessel with contaminated water and then keeping the vessel in full-strength sunlight for several hours to inactivate pathogenic microbes. Using various types of bottles and containers, Acra and co-workers at the University of Beirut demonstrated that sewage-contaminated oral rehydration solutions were able to pass the zero coliform test after one hour of illumination in full-strength sunlight (Acra *et al.*, 1980, 1984). Subsequently, their laboratory and field experiments demonstrated that solar radiation can inactivate a wide range of microorganisms including fecal indicator bacteria such as *Escherichia coli*, water-borne pathogenic bacteria such as *Salmonella typhi* and *Shigella flexneri*, along with various yeasts and molds (Acra *et al.*, 1984). These studies generated interest in the use of sunlight for water treatment, acting as a stimulus for further research studies into batch-process solar disinfection, most notably those of (i) the Integrated Rural Energy Systems Association (INRESA), located at the Brace Research Institute, Montreal (Lawand *et al.*, 1988; 1997), (ii) the Swiss Federal Insitute for Environmental Science and Technology/Department of Water and Sanitation in Developing Countries (EAWAG/SANDEC), Duebendorf (Sommer, *et al.*, 1997; Wegelin, 1999; Wegelin and Sommer, 1998; Wegelin *et al.*, 1994), and (iii) the Royal College of Surgeons in Ireland (Joyce *et al.*, 1996; Kehoe *et al.*, 2001; McGuigan *et al.*, 1998, 1999). Continuous-flow solar water treatment systems have also been developed and evaluated, both by Acra and colleagues (e.g., Acra *et al.*, 1990) and by other researchers (e.g., Fjendbo-Jørgensen *et al.*, 1998; Saitoh and El-Ghetany, 1999, 2002; Wegelin and Sommer, 1998), as detailed in Section V.

While the focus of this review is solar water treatment, it is also worth noting that sunlight is an important factor responsible for the inactivation of fecal bacteria in many natural environments, including fresh water (Barcina *et al.*, 1997; Dan *et al.*, 1997; Kussovski *et al.*, 2001) and sea water (e.g., Barcina *et al.*, 1990; Davies and Evison, 1991; Sinton *et al.*, 1999), bathing waters (e.g., Mascher *et al.*, 2003), and waste waters (e.g., Benchokroun *et al.*, 2003; Mezrioui *et al.*, 1995; Sinton *et al.*, 2002). The most extensive research studies on solar disinfection have been carried out using bacteria, especially fecal indicators and water-borne pathogens, and these will be used to illustrate

the general principles involved. However, examples will be given for other microbes where appropriate.

II. Mechanisms of Solar Disinfection

A. Optical Inactivation

Although sunlight may cause direct damage to biomolecules, such as that seen when UVB radiation is absorbed by DNA (e.g., Jaegger, 1985), it is more common for solar UV and visible light to cause indirect damage, being absorbed by photosensitiser molecules, which are then raised to an excited state. Such photosensitizers may be found either within microbial cells (e.g., porphyrins, flavins and photosynthetic pigments) (Curtis *et al.*, 1992a) or in the surrounding water (e.g., humic substances in natural waters) (Voelker *et al.*, 1997). An excited photosensitizer may then react directly with cellular biomolecules (type I reaction), or more commonly, with molecular oxygen (type II reaction). The latter leads to the production of various reactive oxygen species (ROS) including singlet oxygen, superoxide, and hydroxyl radicals, along with hydrogen peroxide (for details, see Foyer *et al.*, 1994; Whitelam and Codd, 1986). ROS generated by solar irradiation will then react with cellular constituents, including DNA, proteins and cell membrane components, especially membrane lipids (Gourmelon *et al.*, 1994), leading to the inactivation of the cell (e.g., because of increased permeability and/or the disruption of transmembrane ion gradients) (Bose and Chatterjee, 1995; Futsaether *et al.*, 1995). Oxygen-dependent type II photoreactions are likely to form the major component of the optical component of solar inactivation as a result of ROS-induced membrane lipid peroxidation (Bose and Chatterjee, 1994; Davies-Colley *et al.*, 2000) and DNA damage (Jeffrey *et al.*, 1996; Yonezawa and Nichioka, 1999). While cellular antioxidant systems exist to counter the production of ROS, including superoxide dismutase (e.g., Lu *et al.*, 2003; Rao and Sureshkumar, 2000) and catalase (e.g., Hillar *et al.*, 1999; Switala *et al.*, 1999), such antioxidant defense systems are also known to be light-sensitive (e.g., Kapuscinski and Mitchell, 1981).

It is generally accepted that optical effects typically account for the main component of solar disinfection, especially in non-turbid waters, thus Acra *et al.* (1984) attributed 70% of the inhibitory effects of sunlight to the optical properties of solar UV radiation. Not surprisingly, optical inactivation is influenced markedly by the level of dissolved oxygen in the treated water (Reed, 1996), being optimum under oxygen-saturated conditions, as discussed in detail in Section III.A.3.

B. Thermal Inactivation

Here, the absorption of sunlight, especially solar infrared radiation, raises the temperature of the water to a point where microbes are inactivated, in a process often termed *solar pasteurization*, by analogy with commercial pasteurization (e.g., Wegelin and Sommer, 1998). Thus simple batch-process solar pasteurization has been carried out by using a solar box cooker and black-painted container, showing that fecal coliforms are inactivated at water temperatures of 60 °C or greater (Ciochetti and Metcalfe, 1984). Small-scale solar pasteurizers are available commercially (e.g., Anon. 2003a). Such systems are based entirely on thermal inactivation, which is enhanced by using non-transparent black containers, maximizing the absorbance of infra-red radiation. In the early studies of Acra *et al.* (1980, 1984), heat was not considered to play a significant role, as the measured temperature increases were small, and well below the temperatures required to inactivate bacteria.

Solar water distillation systems also rely on the heating effects of infra-red radiation to evaporate water, with the resulting condensate being free of microbial and chemical contamination from the original source (Cappelletti, 2002; Simate, 2001). Solar distillation requires considerably more energy input that solar pasteurization, since the operating temperature of the water is higher in the former case. However, a solar still also provides a means of removing chemical contamination, including desalination of sea water to produce potable water (Garcia-Rodriguez and Gomez-Camacho, 2001). Further details of commercial systems are given by Rolla (1998), while continuous-flow systems are discussed in Section V.

C. Interaction Between Optical and Thermal Effects

Several research studies have reported a synergy between optical and thermal inactivation. Thus Tyrell (1976) showed a synergistic effect of UV radiation and heat in the inactivation of *E. coli*, and Wegelin *et al.* (1994) subsequently demonstrated that temperatures above 50 °C result in a three-fold decrease in the UVA radiation dose required to inactivate *E. coli*, with even more striking effects for bacteriophages and enteroviruses. McGuigan *et al.* (1998) showed synergy at temperatures above 45 °C, where the combined effects of simulated sunlight and heat resulted in a greater rate of inactivation of *E. coli* than that predicted from the rates obtained by using each factor in isolation.

Lawand *et al.* (1997) also noted a synergistic effect of solar radiation and heat on fecal coliforms in contaminated water at temperatures

above 40 °C, suggesting that clear containers should be placed on a dark surface to enhance this effect. Using similar reasoning, Sommer *et al.* (1997) and Wegelin and Sommer (1998) have recommended that the backs of solar disinfection containers should be painted black, to increase the thermal effect. In contrast, Kehoe *et al.* (2001) have shown that the inactivation of *E. coli* can be enhanced by a factor of almost two-fold by adding an aluminium foil backing to the containers to reflect UV and visible light, thereby enhancing the optical component of the process. Comparative trials of foil-backed and black-backed containers have yet to be carried out; it will be important to evaulate both types of containers under different weather conditions, as absorptive, black-backed containers might be expected to give the best effects in full-strength sunlight, where thermal effects are likely to raise the water temperature to 45 °C and above, whereas reflective, foil-backed containers might be more effective in suboptimal sunlight and cloudy conditions, where thermal effects will be greatly reduced and where optical (UV-mediated) inactivation is most important.

III. Laboratory Studies (Batch-Process Systems)

The effects of various factors that might influence the efficacy of solar inactivation have been investigated under controlled laboratory conditions.

A. Chemical Aspects

1. *Organic Compounds*

The presence of dissolved organic compounds such as humic acids may result in the enhancement of solar inactivation as a consequence of their action as photosensitisers. However, such compounds may also absorb sunlight and thereby lead to a reduced inactivation. In natural waters, the inhibitory effects of such dissolved organic compounds will be a trade-off between the positive effects caused by ROS production and negative effects caused by absorption of radiation. While Curtis *et al.* (1992a) have described enhanced solar inactivation in the presence of dissolved humic substances, several other studies have concluded that such compounds reduce the inhibitory effects of sunlight (e.g., Davies and Evison, 1991; Kehoe *et al.*, 2001; Reed, 1997a; Vicars, 1999 and Wegelin *et al.*, 1994), indicating that the primary effect is often due to decreased transmittance rather than enhanced photosensitization. Similarly, while polyaromatic

hydrocarbon pollutants have been shown to enhance phototoxicity in laboratory studies (e.g., Gala and Giesy, 1992), their ecological significance in natural waters has been dismissed as irrelevant (MacDonald and Chapman, 2002). The specific effects of added photosensitizers is considered further in Section VI.

2. *Inorganic Compounds*

Several studies have demonstrated a synergistic interaction between dissolved salts and solar illumination (e.g., Davies and Evison, 1991; Vicars, 1999), though this is most pronounced at the high salt concentrations found in sea water and is likely to be less significant than organic compounds at the salt concentrations found in most natural fresh waters. Inorganic compounds may also affect the pH of the water to be treated: alkaline conditions have been shown to increase the sensitivity of fecal bacteria to sunlight, perhaps as a result of membrane damage, thereby affecting intracellular pH homeostasis (Curtis *et al.*, 1992a,b).

3. *Dissolved Oxygen*

Downes and Blunt (1888) first demonstrated that oxygen was an essential requirement for this inhibitory effect of sunlight, with ''light and oxygen together accomplishing what neither can do alone.'' However, in the same year, Tyndall (1888) reported variable results in attempting to sterilize nutrient infusions by using sunlight, questioning the antimicrobial effectiveness of solar radiation, though the oxygen status of the solutions used in his experiments was not considered. Similarly, while some of the early studies of solar disinfection took no account of the level of aeration of the water under treatment and gave inconsistent results (e.g., Miller, 1988; MacKenzie *et al.*, 1992), subsequent research has shown that a high level of dissolved oxygen is an essential pre-requisite for the rapid inactivation of *Escherichia coli* and *Enterococcus faecalis* in sunlight (Reed, 1996, 1997b). Similar results have been reported for the effects of oxygen on solar disinfection of fecal coliforms and fecal streptococci in natural waters and in waste waters, where the level of dissolved oxygen is often far below the air-saturation value (Acher, 1997; Acher *et al.*, 1994; Meyer, 2001; Meyer and Reed, 2001; Reed *et al.*, 2000). Taken together, these studies have shown that the rate of inactivation of fecal bacteria exposed to sunlight can be 4–8 times faster in oxygenated water as compared with deoxygenated water, demonstrating that photooxidation, with the resultant production of ROS, is the principal reason for the rapid decrease in bacterial counts in water of low turbidity.

Reed (1997c) proposed a four-stage procedure for batch-process solar disinfection, to maximize the photo-oxidative effect, involving:

1. filling the containers, but leaving an air space of up to one-quarter of the volume;
2. vigorously shaking the part-filled container, to ensure that the water becomes oxygen-saturated;
3. exposing the bottles to sunlight for a day, with additional shaking during the day if the water is likely to show a lowering of the dissolved oxygen concentration caused by the respiratory activities of indigenous microbes;
4. storage overnight, allowing the water to cool down before consumption the following day.

Kehoe *et al.* (2001) have questioned the value of regular shaking of the containers during illumination, demonstrating that it made little difference to the inactivation times for *E. coli* in field experiments in Malaysia, and that regular agitation was detrimental to solar disinfection in controlled laboratory experiments where there was a substantial rise in temperature (to over 50 °C), since it promoted the release of dissolved oxygen from the treated water as the temperature was raised. However, the value of additional shaking still remains to be determined for natural waters that have a high rate of oxygen consumption because of the presence of a significant indigenous microflora and assimilable dissolved organic carbon compounds.

B. Physical Aspects

1. *Light Quality and Intensity*

Acra *et al.* (1984) showed that the destruction of coliform bacteria was most efficient at wavelengths of 290–350 nm, corresponding to the UVB and UVA regions of the spectrum, though Curtis *et al.* (1992a) demonstrated that longer wavelengths of up to 700 nm may also inactivate bacteria if exogenous photosensitizers are present in the water. Davies-Colley *et al.* (1997) have shown that solar UVB is most important in the inactivation of *E. coli*, whereas UVB, UVA and short-wavelength visible radiation all contribute to the inactivation of *Enterococcus* spp. The inhibitory effects of UVA and violet light were also demonstrated by Wegelin *et al.* (1994), with a synergistic interaction between light in these two wavebands for *E. coli*, but not for *Enterococcus faecalis*.

There is a general consensus that effective solar disinfection requires around 3–5 hours of strong sunlight at an intensity above 500 W m^{-2}

(Oates *et al.*, 2003). Such intensities are achieved most readily in semi-arid regions between 15 and 35 degrees from the equator, where there is over 3000 h sunshine per year, with those areas lying between the equator and 15 degrees, having around 2500 h sunshine per year being the next most favorable locations (Acra *et al.*, 1984). Given that most developing countries fall within these broad areas, they are well-placed to exploit solar disinfection (Anon, 2003b).

2. *Temperature*

While the synergistic effects of thermal and optical inactivation at temperatures above 40–50 °C were noted in Section II, C, below this threshold, temperature change has a minimal effect on solar inactivation. Thus Wegelin *et al.* (1994) observed that the survival of *E. coli* exposed to solar UV remains unchanged between 12 °C and 40 °C. It is possible that such results may be explained, in part, by the decrease in oxygen solubility that accompanies a rise in water temperature (e.g., Green and Carritt, 1967), with the negative effects of a lower oxygen status cancelling out the expected enhanced inactivation caused by increased temperature. In contrast, the inactivation of viruses has been reported to increase steadily over the range from 20 °C to 50 °C (Wegelin *et al.*, 1994).

3. *Type of Container*

The early studies of Acra and coworkers mostly used glass containers, recommending either clear or blue glass to allow the greatest transmission of UV and short-wavelength visible radiation. For example, clear glass drinking water jugs with a spout designed to allow the user to pour a stream of water directly into the mouth are widely available in Arab countries, offering the added advantage of avoiding post-collection contamination of the spout of the vessel during use (Acra *et al.*, 1984). Subsequent research has also shown that clear glass bottles can give slightly faster rates of inactivation than plastic bottles, though the difference in rate is typically quite small (e.g., Duffy *et al.*, 2003; Shah *et al.* 1996; Sommer, *et al.*, 1997). In contrast, plastic (polyethylene terephthalate, PET) bottles have been used under field conditions, often because they are more readily available or less liable to break (Conroy *et al.*, 1996, 1999). Specially designed plastic bags can also be used (Dold, 2001), giving maximum transmission of UV and visible radiation because of the reduced wall thickness of the bag (e.g., Lawand *et al.*, 1997; Sommer *et al.*, 1997). However, they are less practical, especially for larger volumes, and they may cause the water to taste ''plastic'' (Anon, 2003b). A further disadvantage is the potential

leaching of plasticizers and formation of toxic photoproducts in the treated water. However, Wegelin *et al.* (2001) have shown that such reactions occur only at the outer surface of illuminated plastic (PET) bottles, with no photoproducts or plasticizers being detected in the water contained within the bottles.

Wegelin and De Stoop (1999) have provided a preliminary report on the comparative use of recycled glass bottles and purpose-made plastic PET bottles for solar water treatment in Ethiopia, noting that plastic bottles quickly become scratched during use, suggesting that they will need to be replaced every 4–12 months, according to the degree of wear. In contrast, there is already a market for empty glass spirit bottles for general household usage, which may enhance the acceptability and applicability of solar water treatment to rural villagers.

4. *Turbidity*

Acra *et al.* (1984; 1990), Wegelin *et al.* (1994) and Kehoe *et al.* (2001) have investigated the effects of increasing turbidity on the efficiency of solar disinfection, concluding that the rate of bacterial inactivation decreases as the turbidity is raised to around 300 NTU and recommending that water with a turbidity of >300 NTU should be filtered. Reed (1997a) has shown that the positive effects of dissolved oxygen decrease with increasing turbidity, with a three-fold difference between the rate of inactivation under aerobic and anaerobic conditions in water at 10 NTU, decreasing to less than two-fold at 800 NTU. Such results suggest that turbid water should be filtered or clarified before exposure to sunlight, to maximize the effectiveness of solar photooxidation. One approach is to use a simple, small-scale rapid sand filtration system such as that shown in Fig. 1. Alternatively, the water could be treated by using a clarifier (flocculation) such as ''rauwaq'' clay (Heber, 1985) or with a natural coagulant, such as that found in the seeds of *Moringa oleifera* (Okuda *et al.*, 2001) or *Strychnos potatorum* (Adinolfi *et al.*, 1994). It is also worth noting that such clarification would have the added benefit of reducing the initial microbial population prior to solar irradiation. Reed (1997a) has recommended a three-stage approach to solar water treatment under conditions in which the water has a high level of turbidity and/or color, namely:

1. Clarification—by sedimentation, filtration or coagulation/flocculation.
2. Oxygenation—by vigorous mixing before exposure to sunlight.
3. Illumination—in full-strength sunlight, for as long as possible (a whole day).

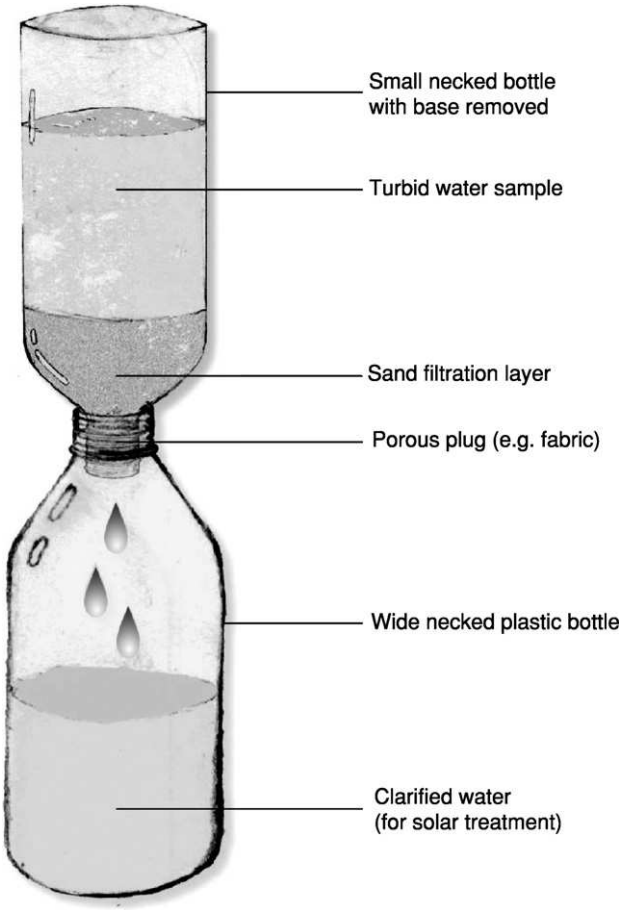

FIG. 1. Simple, small-scale sand filtration system for clarification of turbid water prior to solar disinfection.

However, it is worth noting that Joyce *et al.* (1996) have shown that in highly turbid waters (>200 NTU) such as that used for drinking purposes in rural Kenya, thermal effects resulting from the absorption of visible and infra-red radiation by the suspended particles can raise the water temperature to 55 °C and that this temperature can then account for the complete disinfection of a suspension of *E. coli* within 7 h in the absence of any optical effects. In waters of lower turbidity, the beneficial effects of increased temperature are likely to be far less important than the negative effects resulting from a decrease in penetration of the inactivating radiation (Section II).

C. Microbiological Aspects

The most extensive studies of the dynamics of solar disinfection have been carried out by using bacteria, especially with pure cultures of the fecal indicator bacterium *E. coli*. Typical inactivation curves show an exponential decrease in the bacterial count against time, often with an initial shoulder or plateau, lasting 0.5–2 hours, corresponding to a delay in the inactivation process (Fig. 2). This shoulder is most marked in stationary phase cells (Reed, 1997b) and is generally interpreted in terms of a multiple target model of inactivation (Davies-Colley *et al.*, 1994; Wegelin *et al.*, 1994). After this initial shoulder, the inactivation kinetics generally follow a single-exponential decay function, giving a straight line on a log-linear graph (e.g., Reed, 1997b; Wegelin *et al.*, 1994). However, this is not always the case, and McGuigan *et al.* (1998) have described solar inactivation kinetics of a Kenyan isolate *of E. coli* in terms of a double-exponential decay function involving a light-sensitive and a light-resistant sub-population that gives a non-linear relationship, especially at high irradiances. Salih (2003) has also proposed a more complex model based on the combined effects of (i) exposure and (ii) bacterial load.

A widely used means of representing the exponential decay component of the inactivation process is to calculate the T_{90} value (i.e., the time required to reduce the plate count by 90%) (Guillard *et al.*, 1997).

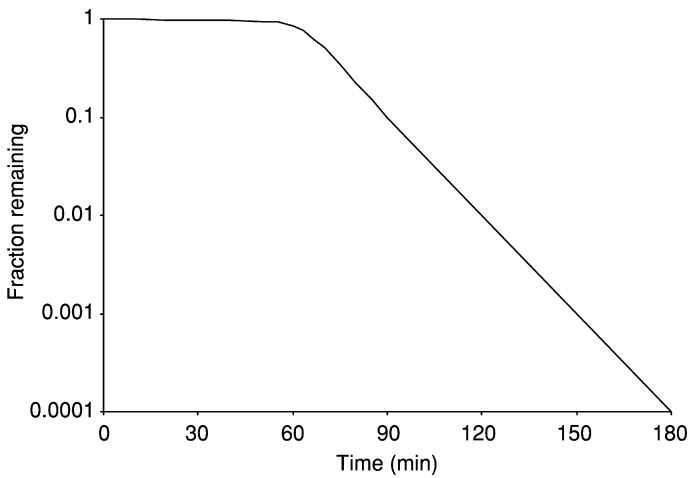

Fig. 2. Representative example of a solar inactivation curve, showing the initial shoulder (approx. 60 min.) and subsequent log-linear exponential decline (T_{90} approx. 30 min.).

Table I shows some representative data for the T_{90} values of a range of fecal bacteria and water-borne pathogens when illuminated either in natural sunlight or in simulated sunlight. Although no single value applies to a particular bacterium, most of the T_{90} values lie between 30 min and 120 min. Of course, such T_{90} values will be influenced by the various chemical and physical factors listed above, and there may also be between-batch variability when experiments are repeated (Davies-Colley *et al.*, 1994). However, Table I provides an indication that a full day of sunlight should be sufficient to inactivate 99.9% of all of the bacteria listed, even with an initial lag period of 1–2 h.

There is considerably less information on the inactivation of other microbes. Wegelin *et al.* (1994) have shown that coliphage f2 and bovine rotavirus are inactivated by a similar amount of light to that required for *E. coli*, whereas encephalomyocarditis virus needed twice the dose, demonstrating the virucidal effects of natural and simulated sunlight. Acra *et al.* (1984) report inactivation of a range of fungi,

TABLE I

T_{90} Values of Various Bacteria Exposed to Natural/Simulated Sunlight

Microbe	T_{90} (min)	Reference
Escherichia coli	84	Evison (1988)
	33	Reed (1996)
	38	Acra *et al.* (1990)
Enterococcus faecalis	30	Reed (1996)
	53	Acra *et al.* (1990)
Fecal coliforms	34–180	Gameson and Gould (1975)
	70	Reed (1996)
	28–38	Fujioka and Narikawa (1982)
	60–120	Solic and Krstulovic (1992)
	50	Acra *et al.* (1990)
Fecal streptococci	140	Evison (1988)
	>120	Fujioka and Narikawa (1982)
	65	Reed (1996)
Shigella sonnei	96	Evison (1988)
Shigella flexneri	67	Kehoe (2001)
Shigella dysenteriae	15	Kehoe (2001)
Vibrio cholerae	35	Sommer *et al.* (1997)
	171	Kehoe (2001)

including *Aspergillus niger, Aspergillus flavus, Candida* sp. and *Geotrichum* sp. within 3 hours, and *Penicillium* sp. within 6–8 hours. Cysts of *Giardia* spp. and *Entamoeba histolytica* can be inactivated by the thermal effects of sunlight (e.g., within 10 min at 56 °C) (Ciochetti and Metcalfe, 1984), though such temperatures are only readily achieved in prolonged strong sunlight under conditions in which the thermal effects are boosted (e.g., by using absorptive black surfaces, or a solar hot box cooker). Optical effects alone are unlikely to inactivate protozoan cysts, as shown for *Acanthamoeba polyphaga* (Lonnen *et al.*, 2004).

IV. Field Studies

There have been few quantitative studies on the effectiveness of solar disinfection under field conditions. The most rigorous experiments have been carried out in Maasai villages in Kajiado province, Kenya (McGuigan *et al.*, 1999). Controlled, randomized field trials were initially carried out with 206 Maasai children, aged 5–16 years. All children were instructed to fill plastic water bottles from the same (contaminated) drinking water source, with half of them keeping their bottles in full sunlight, on the roof of their huts (solar disinfection treatment group), while the other half kept their bottles indoors (dark control group). Children who drank solar-disinfected water showed a 10% reduction in the incidence of diarrheal disease and a 24% decrease in severe diarrhea (Conroy *et al.*, 1996). A year-long follow-up study of 349 children aged 5 or under showed a similar reduction in severe diarrhea, demonstrating that solar water treatment can significantly reduce morbidity in communities with no other means of treating contaminated drinking water (Conroy *et al.*, 1999).

A subsequent cholera epidemic in the same community provided further information on the protective effect of solar disinfection. While there was no significant difference in the risk of cholera in adults or older children (6 years or older), only 3 cholera cases were seen from 155 young children (5 years or under) who drank solar disinfected water as compared with 20 cases out of 144 young children in the control group, giving an odds ratio of 0.12 (Conroy *et al.*, 2001). Given the global significance of *Vibrio cholerae* and similar water-borne pathogenic bacteria (Colwell, 1996; Lipp *et al.*, 2002; Sharma *et al.*, 2003), this study provides clear evidence of the effectiveness of solar disinfection as a point-of-use intervention strategy for improving the quality of drinking water. The continued use of solar disinfection by these Maasai communities also suggests that the benefits of this

approach have been recognized by the target population and a transition to a sustainable health improvement strategy may have been achieved (McGuigan *et al.*, 1999).

A field study involving seven different developing countries (Bolivia, Burkina Faso, China, Colombia, Indonesia, Thailand, and Togo) showed that solar disinfection could be accepted at the village level as an effective means of water treatment: with 500 households in the initial demonstration phase, after 1 year the number of households using solar disinfection had risen to over 10,000, with 84% stating that they intended to continue using this approach after the project had ended (Wegelin and De Stoop, 1999). The major problem noted was the difficulty in convincing both the target population and their public health officials that exposure to sunlight was sufficient to disinfect the water, in contrast to more conventional treatments (e.g., boiling).

V. Continuous-Flow Systems

One of the earliest demonstrations of a continuous-flow solar water treatment process is that described by Acra *et al.* (1990), with reactor volumes of up to 18 liters and exposure times of 8–66 min, reaching the following conclusions:

- The continuous flow system could achieve 99.9% inactivation of *E. coli* and *Enterococcus faecalis* under operating conditions.
- Sewage-contaminated water gave slower rates of bacterial inactivation than those for suspensions of laboratory isolates of fecal bacteria, suggesting that sewage addition may provide a more realistic test of the effectiveness of solar disinfection than pure cultures.
- No visible growth of microalgae was observed on the inner surfaces of the reactor vessel over a period of several weeks, suggesting that a continuous-flow system could be operated without the need for a laborious cleaning regime.

A low-cost compound parabolic collector for solar UV disinfection has been developed and tested by Vidal and Diaz (2000), giving a continuous daily throughput of around 50 liters $m^{-2}h^{-1}$, with successful inactivation of *E. coli* and *E. faecalis*. In contrast, Fjendbo-Jørgensen *et al.* (1998) have described a flow-through solar water treatment system based on direct heating, achieving a minimum water temperature of 65 °C during thermal treatment and using a thermostatically controlled valve to prevent water from being withdrawn until this temperature is reached. This system can achieve a reduction in standard

aerobic plate count of 1000-fold during use, producing treated water at a rate of around 50 liters $m^{-2}d^{-1}$. A similar thermally controlled system has been described by Saitoh and E1-Ghetany (1999; 2002), based around a hot box solar cooker, giving an acceptable performance under partially cloudy conditions, as well as in full sunlight.

Sommer *et al.* (1997) compare the effectiveness of a flow-through system based on a combination of optical and thermal inactivation (minimum temperature 50 °C) with that for thermal inactivation (minimum temperature 70 °C); both systems were effective under clear skies, at flow rates up to 45.6 liters h^{-1} (combined system) or 64.3 liters h^{-1} (solar pasteurization system) but with increasing problems of flow maintenance under cloudy conditions, especially for the thermal system. They concluded that a combined optical plus thermal system is likely to provide the most promising means of developing an effective flow-through system, and this has been further evaluated under field conditions, showing the practical application of this approach (Wegelin and Sommer, 1998).

VI. Photocatalytic Solar Water Treatment Systems

The use of catalysts to enhance the inactivation of microbes has been the subject of extensive investigation. Photosensitizers such as methylene blue or rose bengal are known to enhance the production of ROS in aqueous solution and thereby increase the antimicrobial effects of light (e.g., Chilvers *et al.*, 1999; Wegelin *et al.*, 1994). While Acra and Ayoub (1997) have described a solar photocatalytic water treatment system based on the combined use of methylene blue as a photosensitizer, along with chlorine to decolorize the dye prior to consumption of the treated water, this approach is far more complex that standard batch-process solar disinfection and is relatively impractical under field conditions, since it requires the addition of defined amounts of two different reagents, neither of which is likely to be readily available to those without access to treated water in developing countries. However, such photosensitizers may have applications for waste water treatment (e.g., with dye removal by adsorption onto bentonite clay) (Acher, 1984; Acher and Juven, 1977; Gerba *et al.*, 1977).

In contrast, the semiconductor titanium dioxide, typically in the anatase mineral form, can be used as a stable heterogèneous photocatalyst since excitation of TiO_2 by short-wavelength light (<385 nm) leads to the generation of ROS, principally hydroxyl radicals (Harper *et al.*, 2001; Ollis *et al.*, 1991). One of the earliest studies was that of Matsunaga *et al.* (1985), who showed that water containing the

Gram-negative bacterium *E. coli*, the Gram-positive bacterium *Lactobacillus acidophilus*, or the yeast *Saccharomyces cerevisiae* could be sterilized within 60–120 min when illuminated by a metal halide lamp in the presence of a suspension of platinum-loaded TiO_2 particles. Subsequently these studies were extended, demonstrating that similar effects could be obtained with a continuous-flow treatment system with TiO_2 immobilized on acetylcellulose membranes (Matsunaga *et al.*, 1988). Rapid inactivation of *E. coli* in water containing particulate TiO_2 has been confirmed in subsequent studies (e.g., Ireland *et al.*, 1993). Photocatalytic inactivation has also been reported for several other microbes, including bacteria, fungi, viruses, and some protozoa, as shown in Table II.

Sunlight-assisted photocatalysis with a supension of TiO_2 particles has been demonstrated by Wei *et al.* (1994), who showed that longer-wavelength radiation (>385 nm) is effective in the inactivation of *E. coli*, illustrating the potential applicability of solar photocatalytic disinfection of water as an alternative to chemical treatment. Subsequently, Block and Goswami (1995) used simulated sunlight to demonstrate that TiO_2-enhanced photoinactivation is decreased under alkaline conditions (pH 8.6) and in the presence of either inorganic or organic compounds but is improved by continuous mixing. Rincón *et al.* (2001) have also shown a decrease in the effectiveness of TiO_2 photocatalysis in the presence of organic disinfectant byproducts, including dihydroxybenzene, hydroquinine, resorscinol, and catechol.

Salih (2002) has demonstrated that TiO_2 enhances the solar inactivation of *E. coli*, irrespective of whether it is used as a particulate suspension or in immobilized form within the test container. In the same study, the addition of quenchers of hydroxyl radicals, such as dimethyl sulphoxide and cysteine was found to abolish the TiO_2-dependent component, supporting a direct role for their involvement in the inactivation process. Using mannitol as a hydroxyl scavenger, Kikuchi *et al.* (1997) have also demonstrated that these radicals, generated at the surface of the semiconductor, are important initial products but that the subsequent production of H_2O_2 is likely to play a major role in the process, since the effect of TiO_2 was strongly inhibited by the addition of catalase. In terms of practical application, immobilized systems offer the advantage that the water remains uncontaminated by particulate TiO_2, in contrast to suspended particulate systems, in which filtration or centrifugation is required to separate the catalyst and water after illumination. Duffy *et al.* (2004) have shown that a simple batch-process solar photocatalytic disinfection system can be achieved by using a glass or plastic bottle fitted with an insert comprising a flat

TABLE II

MICROBES INACTIVATED BY TiO_2-ASSISTED PHOTOCATALYTIC SOLAR DISINFECTION

Microbe	Reference
Bacteria	
Enterobacter cloacae	Ibáñez *et al.* (2003)
Enterococcus faecalis	Herrera Melián *et al.* (2000)
Escherichia coli	Zhang *et al*, (1994)
	Bekbölet (1997)
	Blake *et al.* (1999)
	Choi and Kim (2000)
	Wist *et al.* (2002)
	Sun *et al.* (2003)
Fecal coliform bacteria	Watts *et al.* (1995)
Listeria monocytogenes	Kim *et al.* (2003)
Pseudomonas stuzeri	Biguzzi and Shama (1994)
Streptococcus mutans	Onoda *et al.* (1988)
	Morioka *et al.* (1988)
	Saito *et al.*, 1992)
Salmonella typhimurium	Ibáñez *et al.* (2003)
Vibrio parahaemolyticus	Kim *et al.* (2003)
Viruses	
Poliovirus	Watts *et al.* (1994)
RNA coliphage Qβ	Lee *et al.* (1997)
Fungi	
Candida albicans	Lonnen *et al.* (2004)
Fusarium solani	Lonnen *et al.* (2004)
Protozoa	
Acanthamoeba polyphaga (trophozoites*)	Lonnen *et al.* (2004)
Cryptosporidium parvum (oocysts)	Ogaki *et al.* (2000)
	Otaki *et al.* (2000)

*Note that cysts of *A. polyphaga* are insensitive under the same conditions.

plastic (PET) sheet coated with TiO_2 on the upper-face, demonstrating that this may be an appropriate and affordable technology for use in urban and semi-urban areas of developing countries. More complex continuous-flow UV disinfection systems have also been described, for the treatment of wastewaters (Li *et al.*, 1996).

The effectiveness of TiO_2-driven photocatalysis can be enhanced by the inclusion of other metals or metallic salts, including those of tungsten (Lettmann *et al.*, 2001; Li *et al.*, 2001), ruthenium (Fung *et al.*, 2003), silver (Dobosz and Sobczynski, 2003), and iron (Sun *et al.*, 2003) or by coating the surface with a photosensitizing dye (e.g., Cho *et al.*, 2001; Lobedank *et al.*, 1997). An alternative approach has been described by Butterfield *et al.* (1997), who showed that the application of a small positive charge (electric field enhancement) to the TiO_2 surface boosts the production of hydroxyl radicals and thereby greatly improves the catalytic performance in comparison with conventional TiO_2 systems. This is particularly effective against more resistant microbes (e.g., spores of *Clostridium perfringens*) (Butterfield *et al.*, 1997) and oocysts of *Cryptosporidium* (Curtis *et al.*, 2002). The process has been termed *photoelectrocatalytic disinfection*, to distinguish it from the conventional photocatalytic process (Christiansen *et al.*, 2003).

The underlying mechanism responsible for the photocatalytic inactivation of *E. coli* has been investigated by Maness *et al.* (1999a,b), who have provided evidence that peroxidation of membrane lipids is a significant factor, with evidence of the enhanced accumulation of malondialdehyde (MDA) and decreased respiratory activity following illumination of *E. coli* in the presence of TiO_2, Supporting evidence is available for *Streptococcus sobrinus* (Saito *et al.*, 1992), where leakage of intracellular K^+ ions indicates the cell membrane as a site of significant damage. Huang *et al.* (2000) have used *o*-nitrophenol β-D-galactoside (ONPG) as a probe for cell membrane integrity, demonstrating that short-term exposure of *E. coli* to TiO_2 and UV light results in an increase in cell membrane permeability to ONPG, followed by the leakage of intracellular β-galactosidase from the cell after 20 minutes. Taken together, these studies indicate that membrane lipid peroxidation, resulting from TiO_2-catalyzed ROS such as hydroxyl radicals and hydrogen peroxide plays a key role in the inactivation process.

VII. Death, Dormancy, or Damage?

So far this review has carefully avoided describing the inactivation of microbes by illumination in sunlight in terms of their death, which is the traditional view of a decrease in the plate count of bacteria subjected to extreme environmental conditions. This is because bacterial viability has been the subject of much debate and conjecture in recent years, with many researchers questioning the conventional view that a decrease in the culturable count corresponds to a loss of

viability (for an overview of this debate, see Barer and Harwood, 1999). For example, Barcina *et al.* (1990) have interpretated differences between acridine orange direct microscopic counts and culture-based plate counts of bacteria exposed to light in terms of a survival strategy for fecal bacteria, involving a dormant, non-culturable, ''somnicell'' state in which bacteria such as *E. coli* and *E. faecalis* are unable to form colonies on standard bacteriological media yet retain their viability. The so-called ''viable but non-culturable'' (VNC) hypothesis (e.g., Roszak and Colwell, 1987; Xu *et al.*, 1982) proposes that this may represent a response to environmental stress in some bacteria, in which cells may become inactive, or dormant, to an extent where they do not form surface colonies on conventional agar-based media yet may remain alive, being detectable *via* methods based on metabolic activity (Barer *et al.*, 1993). If true, then such a survival strategy could compromise the validity of conventional microbiological counting procedures for testing water quality (e.g., Clesceri *et al.*, 1998).

Another aspect of the debate concerns the use of conventional selective media, for example in the enumeration of a target group of microbes in a natural water sample containing a mixed microflora. For example, Dawe and Penrose (1978) concluded that coliform bacteria exposed to sunlight in surface seawater are not killed outright but are debilitated to the extent that they are unable to form colonies on a selective medium (e.g., eosin-methylene blue agar), although they remain able to grow on non-selective nutrient agar. Similarly, Fujioka and Narikawa (1982) demonstrated that exposure to sunlight damages fecal coliforms and fecal streptococci to the point where they form fewer colonies on selective media (mFC agar and KF agar, respectively) as compared with a non-selective medium (phenol red-lactose agar). Shah *et al.* (1996) have also shown that solar-illuminated *E. coli* may be unable to grow on a selective fecal coliform (mFC) medium yet retain an ability to form colonies on non-selective plate count agar medium. Taken together, these studies demonstrate that conventional selective media for fecal indicator bacteria may give reduced counts or, in extreme cases, may result in false-negative counts when used with sunlight-illuminated cells, casting doubt on some of the earlier data obtained by using selective media (e.g., Gameson and Gould, 1975). In consequence, Shah *et al.* (1996) have recommended that solar disinfection studies should use non-selective agar media to avoid the risk of missing low numbers of such injured bacteria. Such findings are also consistent with the wider view that exposure of fecal bacteria to the conditions found in natural waters is likely to cause sub-lethal injury, with the resultant loss of counts on selective media (e.g., Bissonnette *et al.*, 1975; McFeters *et al.*, 1986).

A more recent development concerns the effect of oxygen on the growth of sunlight-illuminated bacteria. To date, most studies of solar disinfection have been carried out by using isolates of *E. coli* enumerated on agar-based media following incubation under conventional aerobic conditions. However, several studies have now shown that it may be necessary to question the validity of this approach; while aerobic conditions are effective in enabling the growth and enumeration of healthy cells, they do not always allow physiologically damaged cells to grow (Bloomfield *et al.* 1998). Specifically, there is evidence that cells of *E. coli* exposed to simulated sunlight show an enhanced sensitivity to ROS during subsequent culture, giving higher counts when grown on media incorporating substances that detoxify ROS; these substances include scavengers of peroxides such as pyruvate and the H_2O_2-degrading enzyme catalase (Kehoe, 2001). Khaengraeng and Reed (2004) have also demonstrated that conventional aerobic plate counts of illuminated *E. coli* are consistently lower than those under conditions where ROS are neutralized, either (i) by the addition of 0.05% w/v sodium pyruvate with subsequent incubation in an anaerobic jar or (ii) by culturing on plates of pre-reduced growth medium within an anaerobic cabinet, indicating that a substantial proportion of such cells are sublethally injured to the point where they are unable to grow under standard aerobic conditions. Recent research has provided a convincing interpretation for such observations, suggesting that sublethal injury can lead to a growth-arrested state in which bacterial cells undergo a process of respiration-induced death when subsequently cultured under aerobic conditions, because of the production of a respiratory burst of intracellular ROS that is uncoupled from growth and which may overwhelm cellular antioxidant defenses systems (Aldsworth *et al.*, 1999; Dodd *et al.*, 1997). Such findings offer a cogent, alternative explanation for the VNC phenomenon, which is mostly based on a comparison of conventional aerobic counting procedures and culture-independent counts (Bloomfield *et al.*, 1998). Similar conclusions have been reached by Bogosian and colleagues, based on studies involving the use of peroxide-scavenging agents (e.g., pyruvate, demonstrating that ROS-sensitive cells can be cultured in the presence of such agents (Barer *et al.*, 2000; Bogosian and Borneuf, 2001; Bogosian *et al.*, 2000). This also provides an explanation of the beneficial effects of added pyruvate in enabling higher counts of injured bacteria on agar-based media (e.g., Kehoe *et al.*, 2004; MacDonald *et al.*, 1983; Sartory, 1995), since hydrogen peroxide is a significant component of respiratory ROS (Valderrama *et al.*, 2002). The inhibitory effects of aerobic culture are also consistent with the

suggestion that the cell membrane is a principal site of photo-oxidative damage (Section II, A), since ROS generated as a result of membrane-associated respiratory processes would lead to further damage in cells sublethally injured by exposure to sunlight.

Taken together, the evidence suggests that sublethal injury of bacterial cells leads to a similar ROS-sensitive state, irrespective of whether the injury is caused by light (Khaengraeng and Reed 2004), heat (Bromberg *et al.*, 1998; George and Peck, 1998; Mizunoe *et al.*, 1999), cold (Bogosian *et al.*, 2000) starvation (Bloomfield *et al.*, 1999), or acid stress (Gnanou Besse 2002). One of the most effective methods of preventing such self-destruction is to culture the organisms in the complete absence of oxygen, forcing them to use anaerobic pathways for energy metabolism; thus Stephens *et al.* (2000) have demonstrated that anaerobic culture can counter the combined effects of (i) neutralization of extracellular ROS generated within the growth medium together with (ii) the prevention of intracellular (respiratory) ROS and that this approach consistently provides the highest counts for sublethally injured *Salmonella*. The results of Kapuscinski and Mitchell (1981), who demonstrated that *E. coli* cells exposed to sunlight gave lower aerobic colony counts on a carbohydrate-rich medium and higher aerobic counts on a minimal medium, can be reinterpreted in terms of the respiration-induced suicide hypothesis, with an increased production of ROS from respiratory carbohydrate metabolism during growth on the nutrient-rich medium, leading to enhanced ROS-induced self-destruction. This has implications for environmental microbiology, where many conventional aerobic plate counting procedures are based on the metabolism of specific carbohydrates as a taxonomic marker (Collins *et al.*, 2003; Walter *et al.*, 1994) (e.g., in the enumeration of coliforms and *E. coli* when using media based on the utilization of lactose) (e.g., Anon, 2002a; Clesceri *et al.*, 1998) and where sublethally damaged cells recovered from environmental samples may then suffer a similar fate when counted under aerobic conditions. The use of selective agents is likely to further compound the problems of aerobic growth of sunlight-injured cells, since it imposes an additional stress on these bacteria during the enumeration procedure (Shah *et al.*, 1996).

A practical consequence of these findings is to place a question mark over those earlier studies of solar disinfection in which aerobic plate counts have been used to determine the extent of bacterial inactivation (e.g., Table I). It would seem appropriate to suggest that future studies of solar water treatment should take into account the potentially inhibitory effects of oxygen, and especially respiration-derived ROS,

during the enumeration procedure (e.g., by making bacterial counts under fully anaerobic conditions, to enumerate damaged cells). Similarly, it is appropriate to consider how the problems associated with selective media may be overcome (e.g., by using agar overlay methods to avoid exposure to selective agents during the intial stages of growth) (e.g., Kang, 2002). However, as a counterpoint, Smith *et al.* (2000) have demonstrated that the pathogenicity of cells *of Salmonella typhimurium* sublethally injured by solar illumination is reduced in comparison to that of their uninjured counterparts, showing a lower infectivity for BALB/c mice (based on aerobic plate counts and most probable number methods) as compared with non-irradiated cells. Such findings suggest for sublethally injured cells that the positive effects associated with reduced infectivity could outweigh the negative implications of a reduced aerobic plate count on conventional selective media, though further work is required to enable this suggestion to be evaluated fully.

A related question is whether sublethally injured cells are able to repair the damage caused by exposure to sunlight when maintained in darkness. While most studies have shown no recovery in the aerobic count of solar-illuminated bacterial suspensions kept for a further 12–24 h in the dark (e.g., Joyce, 1997; Joyce *et al.*, 1996; Oates *et al.*, 2003; Reed, 1997b; Shah *et al.*, 1996; Sommer *et al.*, 1997; Wegelin *et al.*, 1994), there is evidence that in some instances the count may rise on storage in darkness following short-term exposure to simulated sunlight (Rincón and Pulgarin, 2003). Similarly, some studies of TiO_2-based solar photocatalytic disinfection have provided evidence of recovery, with an increase in count following 24 h (e.g., Wist *et al.*, 2002), while other studies have shown no change in count for cells illuminated in the presence of TiO_2 and subsequently maintained in darkness (e.g., Rincón and Pulgarin, 2003). Repair of injury might be expected to reduce the effect of ROS under aerobic conditions, thereby providing one possible explanation for instances in which recovery or regrowth has been observed; this can be resolved only by further experimental studies (e.g., by comparing the count of illuminated cells kept in the dark and enumerated under aerobic conditions with those obtained under conditions in which ROS are neutralized).

VIII. Conclusions and Future Prospects: Practical Implementation of Solar Water Treatment

While solar disinfection is not universally applicable, it may be appropriate under circumstances in which there is no realistic alternative treatment process and where there is an unfulfilled need for safer

water. The following list (adapted from Reed 1997c) provides some instances in which solar disinfection may be useful:

1. Medium to long-term provision, including (i) treated water for people in rural villages and urban shanty communities, who may have access only to sewage-contaminated surface water and (ii) decontaminated water for widely dispersed rural populations where a piped water supply is impractical and where chemical treatment is too costly.
2. Short-term provision, including (i) emergency supply for refugees and in war zones, where conventional water supplies may be unavailable or inoperative (ii) in response to a specific contamination event (e.g., flooding or stormwater overflow), and (iii) in response to contamination with a specific pathogen (e.g., during an outbreak of cholera or bacterial diarrhea).
3. Specific provision for a target group, including (i) treated drinking water for babies and infants, who are most at risk from diarrheal disease (Anon., 2002b; Ribeiro, 2000) and (ii) decontaminated water for the preparation of oral rehydration solution.

The successful use of solar disinfection in reducing the incidence of cholera during field trials in Kenya (Conry *et al.*, 2001) confirms the practical value of this approach, which has been highlighted as a self-sustaining means of treating unsafe water at the point-of-use (Mintz *et al.*, 2001), and the World Health Organization has advocated solar disinfection as a small-scale, low-cost intermediate action (Anon, 2001; Sobsey, 2002). The recent demonstration that conventional batch-process solar disinfection can be modified to take advantage of TiO_2-enhanced photocatalysis, thereby reducing the irradiation dose (Duffy *et al.*, 2003) and/or extending the range of microbes against which it is effective (Lonnen *et al.*, 2003), is a promising avenue for further research and development.

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