ADVANCES IN

APPLIED MICROBIOLOGY

VOLUME 59

ADVANCES IN

Applied Microbiology

VOLUME 59

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ADVANCES IN

Applied Microbiology

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VOLUME 59

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Academic Press is an imprint of Elsevier

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525 B Street, Suite 1900, San Diego, California 92101-4495, USA
84 Theobald's Road, London WC1X 8RR, UK

This book is printed on acid-free paper.

For information on all Academic Press publications
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ISBN-13: 978-0-12-002661-6
ISBN-10: 0-12-002661-9

PRINTED IN THE UNITED STATES OF AMERICA
06 07 08 09 9 8 7 6 5 4 3 2 1

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Biodegradation by Members of the Genus *Rhodococcus*: Biochemistry, Physiology, and Genetic Adaptation

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I. Introduction

It is commonly understood that microorganisms degrade most organic compounds. Indeed it was Stanley Dagley who noted some time ago, ''On thermodynamic grounds, no organic compound can be excluded from serving as a possible energy source for aerobic microorganisms'' (Gibson, 1984). Members of the genus *Rhodococcus* appear to have risen to this considerable challenge and exemplify the principle that diverse genes and catabolic pathways must evolve to match the diversity of organic compounds available. The rhodococci themselves are a very diverse group of bacteria that possess the ability to degrade a large number of organic compounds; particularly many that are recalcitrant and toxic. The basis for this capability lies in their cellular physiology, capacity to adapt to new substrates, and an ability to acquire a wide range of catabolic genes. All of those investigated in detail to date appear to have large and complex genomes and

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 59 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(06)59001-X

have acquired many genes by recombination. Many also possess both large linear plasmids and circular plasmids that contribute greatly to the overall complement of catabolic genes. In addition, there is increasing evidence that multiple pathways and gene homologs are present that further increases *Rhodococcus* catabolic versatility.

The importance of *Rhodococcus* spp. is further acknowledged by the increasing number of reports of these organisms in the literature. This trend reflects an increasing interest in the ability of rhodococci to degrade a wide variety of xenobiotic compounds and their importance as mammalian and plant pathogens. With respect to their environmental significance, metabolic versatility, and potential for biotechnological applications, rhodococci are in some respects similar to the pseudomonads and related bacteria. These share with rhodococci a number of features such as catabolic diversity, a relatively large genome size (approximately 5–6 Mb), and flexible genomes. However, although the mechanisms underlying the flexibility of the *Rhodococcus* genome are not well understood, they appear to differ from those found in pseudomonads (Larkin *et al.*, 1998, 2005). One striking feature is the abundance of large linear plasmids. Other characteristics are the presence of a system that promotes high-frequency–illegitimate recombination and the presence of relatively few transposons (although a number of insertion sequences have been identified). Indeed, other yet unknown events of illegitimate recombination may serve to promote the introgression of DNA in their genomes without the help of mobile genetic elements (de Vries and Wackernagel, 2002). The remarkable ability of members of the genus *Rhodococcus* to degrade many organic compounds, their ability to produce surfactants, and their environmental persistence make them ideal candidates for enhancing the bioremediation of contaminated sites. Many members of the genus have also proved to be of immense use in a wide range of biotransformations such as steroid modifications, enantioselective synthesis, and the production of amides from nitriles. Aspects of their overall metabolic diversity and genetics have been covered previously (Bell *et al.*, 1998; Larkin *et al.,* 1998; O'Brien *et al.,* 2002; van der Geize and Dijkhuizen, 2004; Warhurst and Fewson, 1994). There are also recent reviews (de Carvalho and da Fonseca, 2005; Gurtler *et al.*, 2004; Larkin *et al.*, 2005) of the capabilities of rhodococci and related bacteria. This chapter will focus on some aspects of their biochemical and genetic versatility and further emphasize the importance of these bacteria in environmental applications.

II. General Features of Rhodococci: Taxonomy and Diversity

The genus *Rhodococcus* includes a diverse grouping within the wider group of nocardioform actinomycetes and is common in many environmental niches from soils to fresh water, seawater, plants, and animals (Finnerty, 1992; Larkin *et al.*, 1998; Warhurst and Fewson, 1994). Specifically, the genus *Rhodococcus* belongs to the Actinomycetes, a phylum of the Gram-positive bacteria with high-GC content. Based upon several complete genome sequences, the Actinomycetes appear to form a clade with Deinococcales and Cyanobacteria, whereas the low-GC Gram-positive bacteria are joined only at a deeper node of the phylogenetic tree (Wolf *et al.*, 2001). Together with bacteria of the genus *Nocardia*, those of the genus *Rhodococcus* are members of the *Nocardiaceae* family and belong to the suborder of the *Corynebacterineae* (Stackebrandt *et al.*, 1997). Other well-known families of this suborder include the *Mycobacteriaceae* (*Mycobacterium* genus) and *Corynebacteriaceae* (*Corynebacterium* genus). Earlier classification of *Rhodococcus* strains has often been based upon cell wall structure and for many years their taxonomic position has been somewhat confused. However, since 1989 this appears to have been, in the main, resolved (Goodfellow, 1989). Classification based largely upon cell wall composition (particularly the presence of long-chain mycolic acids) and 16S rRNA sequences (Rainey *et al.*, 1995) has firmly placed *Rhodococcus* taxonomically close to the genus *Nocardia*. However, the adoption of 16S rRNA sequence analysis has also led to considerable changes in actinomycete classification, and it is worth noting that many strains classified in the genus *Rhodococcus* without the aid of 16S rRNA sequence analysis may belong to other genera and that some others, which are currently assigned to the genus *Rhodococcus*, may be classified incorrectly. Some strains originally identified as *Rhodococcus* species were reassigned to the genus *Mycobacterium*, for example, *Rhodococcus chlorophenolicus* (Briglia *et al.*, 1994; Haggblom *et al.*, 1994). *Nocardia corynebacterioides* has been reclassified as *Rhodococcus corynebacterioides* (Yassin and Schaal, 2005).

Rhodococci have been isolated from various environments such as soils, the rhizosphere and surfaces of plants, groundwater, seawater (including deep sea), sediments, and many polluted sites. Many members of the genus *Rhodococcus* are nonpathogenic with the exception of *Rhodococcus fascians* (Goethals *et al.*, 2001) and *Rhodococcus equi* (Kedlaya *et al.*, 2001). Interestingly two strains of *Rhodococcus triatomae* have recently been identified as symbionts in the blood-sucking bug of the genus *Triatoma* (Yassin, 2005).

III. Biodegradation Capabilities

A. Overview of Catabolic Range

Unlike the pseudomonads, the rhodococci in general grow relatively slowly and appear to rely upon persistence for their long-term survival in the environment alongside a remarkable metabolic versatility (Bell *et al.*, 1998; Warhurst and Fewson, 1994). Compounds that are metabolically transformed or degraded include short- and long-chain hydrocarbons (linear or branched, ranging in size from ethylene to paraffins), halogenated hydrocarbons, aromatics (including halogenated and nitro-substituted examples), heteroaromatics, and polycyclic aromatics. Additionally these bacteria play an important role in conversion of plant secondary metabolites in soil and in the rhizosphere. Several plant-derived natural secondary metabolites, such as alkaloids, terpenes, and sterols are known to be transformed or degraded by rhodococci (Cameron *et al.*, 1994; Duetz *et al.*, 2001; Larsen *et al.*, 2002; van der Geize and Dijkhuizen, 2004; van der Geize *et al.*, 2000, 2001, 2002a,b; van der Vlugt-Bergmans and van der Werf, 2001; van der Werf, 2000; van der Werf and Boot, 2000). Pesticides that are degraded by rhodococci include the thiocarbamate and *s*-triazines herbicides (De Schrijver *et al.*, 1997). It is not surprising that their catabolic diversity and environmental niches overlap with those of many other bacteria. Interestingly, this capability has recently been extended to include biodegradation of the quorum-sensing molecules N-acylhomoserine lactones by *R. erythropolis* W2 through a novel oxidoreductase and amidolytic activity (Uroz *et al.*, 2003, 2005). It is intriguing to speculate that this ability could confer a distinct advantage to biodegradative rhodococci in the environment over bacteria that respond to these quorum-sensing molecules. However, it is also notable that rhodococci appear to extend their biodegradative repertoire to attack compounds that could be described as more difficult with respect to recalcitrance and toxicity.

Rhodococci have also have proved to be of use in a range of biotransformations such as steroid modifications, enantioselective synthesis, and the production of amides from nitriles (de Carvalho and da Fonseca, 2005; Warhurst and Fewson, 1994). For example, two distinct oxygenases from *Rhodococcus* strain NCIMB 12038 can produce either the *cis* or *trans* stereoisomers of indandiol (Allen *et al.*, 1997), a key intermediate in the production of Crixivan® (Merck), which is a potent HIV protease inhibitor. The naphthalene *cis*-dihydrodiol dehydrogenase enzyme from the same strain has also been used for the production of catechol-like metabolites in

biotransformation studies (Boyd *et al.*, 2002). The economic significance of rhodococci should not be underestimated and should fuel renewed efforts to define the basis of their physiology and organization of their genetic systems.

B. Physiological Attributes

Rhodococci generally appear to be adapted to tolerate and metabolize many hydrophobic pollutants in the presence of more readily assimilable carbon sources due to their hydrophobic cell surface containing mycolic acids. Alkane-degrading rhodococci also adhere readily to oil droplets (Whyte *et al.*, 1999). Commonly associated with their biodegradative ability is also the production of biosurfactants (Finnerty, 1992; Philp *et al.*, 2002) that can have applications in industrial processes; particularly in soil bioremediation (Lang and Philp, 1998). Surfactants, such as the glycolipids from *Rhodococcus erythropolis*, are more biodegradable and probably less toxic than many of their synthetic counterparts and may offer a better alternative for enhancing the bioremediation of hydrophobic contaminants. Conversely, the production of surfactants by *Rhodococcus* spp. brings about the activated sludge foams that can adversely affect the operation of wastewater treatment plants (Davenport *et al.*, 1998). However, these problems may be more than offset by the positive attributes of rhodococci as there are many biodegradative strains present in wastewater treatment systems. A good example is the range of strains isolated from wastewater treatment plants that are capable of degrading human estrogens (Yoshimoto *et al.*, 2004). Interestingly 16S rDNA sequencing confirmed that three of the strains were *Rhodoccus equi*, while a fourth example was identified as *Rhodococcus zopfii*. They were able to degrade four different estrogens (including 17β-estradiol) into unidentified metabolites. Furthermore and importantly, the degradation products of 17β-estradiol were shown to not have any estrogenic activity against human breast cancer cells.

Another important feature of rhodococci is their ability to continue biodegradation under potentially adverse conditions such as low temperatures, which is important for effective bioremediation in very cold climates (Bej *et al.*, 2000; Kunihiro *et al.*, 2005; Whyte *et al.*, 1999). Recently it has been shown that *Rhodococcus rhodochrous* cells not only survive electrical currents but convert nitriles to their corresponding carboxylic acids more effectively when a direct electric current is applied (Mustacchi *et al.*, 2005a,b).

The ability of rhodococci to utilize many recalcitrant and toxic substrates may also be aided at a physiological level with a general

tolerance to toxic substrates and solvents. For example several *Rhodococcus* strains remain active at desulfurizing fuels when in the presence of a range of solvents and high concentrations of hydrocarbons (Bouchez-Naitali *et al.*, 2004). It has also been noted that rhodococci can tolerate water immiscible solvents, such as dodecane, bis(2-ethylhexyl) phthalate, and toluene, up to 5% v/v, and water miscible solvents, such as ethanol, butanol, and dimethylformamide, up to 50% v/v (de Carvalho *et al.*, 2004). Indeed the capability of *Rhodococcus erythropolis* DCL14 to adapt to tolerate solvents and potentially toxic biotransformation products has been exploited in the biotransformation of terpene (-)-carveol to the valuable flavor compound (-)-carvone (de Carvalho *et al.*, 2005). The unique ability of rhodococci to degrade many volatile compounds, such as aromatic compounds and halogenated alkanes, may also be attributed to their physiological tolerance to these compounds (Erable *et al.*, 2004) and these characteristics make them useful in biofilter treatment processes (Aldric *et al.*, 2005; Erable *et al.*, 2005).

C. Role of Dehalogenases

Although halogenated organic compounds are commonly produced by industry they are often found in nature (Winterton, 2000), and bacteria have evolved to adopt several different enzymatic strategies to remove the halide atom (Fetzner, 1998). Hydrolytic dehalogenation by halohydrolases is very common, however, this is not always the preferred mechanism. For example, the *s*-triazine hydrolase (TrzA) from *Rhodococcus corallinus* (Mulbry *et al.*, 2002) has a very limited substrate range. Instead these compounds may be more commonly attacked by less specific P450 oxygenases as discussed in more detail later. In contrast to this, the *Rhodococcus rhodochrous* NCIMB 13064 1-chloroalkane halidohydrolase DhaA (RrDHL) exhibits a wide specificity; attacking many haloalkanes and secondary alkyl halides (Curragh *et al.*, 1994; Kulakova *et al.*, 1997; Sallis *et al.*, 1990). Not surprisingly, it exhibits structural similarities to the hydrolytic dehalogenase DhlA from *Xanthobacter autotrophicus* GJ10 (Damborsky and Koca, 1999; Damborsky *et al.*, 2001; Newman *et al.*, 1999). However, one notable difference is that DhaA appears to more efficiently dehalogenate secondary alkyl halides than DhlA (Schindler *et al.*, 1999). Both share a similar enzymatic mechanism except that release of the alcohol product is the slowest step in the catalytic cycle compared to release of the halide ion in DhlA (Bosma *et al.*, 2002, 2003).

D. Biodegradation of Nitroaromatic and Heterocyclic Compounds

Nitroaromatic and heterocyclic compounds are important industrial pollutants and appear as common contaminants in wastewaters and soils. The presence of the nitro group makes many of these compounds significantly more recalcitrant than their nonsubstituted counterparts. Furthermore, nitroaromatic compounds and their metabolites are particularly toxic and may be carcinogenic (Spain, 1995). Rhodococci, however, are known to effectively degrade nitroaromatic compounds, such as 4-nitrocatechol (Navratilova *et al.*, 2005) or 2,4,6-trinitrophenol (picric acid), to eliminate nitrite from a dihydride Meisenheimer complex (Hofmann *et al.*, 2004; Rieger *et al.*, 1999). More recently the genes (*npc)* involved in 4-nitrophenol degradation by *Rhodococcus opacus* SAO101 have been characterized and suggest that *npcB* and *npcA* encode a two-component monooxygenase (Kitagawa *et al.*, 2004).

Biodegradation of S- and N-heterocyclic compounds has been the subject of several studies. The biodegradation of quinolone and its chlorinated derivatives (Fuchs *et al.*, 1991) and pyridine (Yoon *et al.*, 2000) by rhodococci has been established. Importantly, *Rhodococcus* strains have been found to be the most efficient at removing sulfur from coal and oil after hydrodesufurization that leads to the release of recalcitrant S-heterocyclic compounds such as benzothiophene (BT), dibenzothiopene (DBT), and 4,6-dimethyldibenzothiophene. It is notable that *Rhodococcus* strains are the only bacteria effective in this respect. However, there are differences in the substrate ranges of various strains. For example, *Rhodococcus* sp. ECRD-1 attacks all isomers of DBT (Prince and Grossman, 2003). In contrast, *Rhodococcus* sp. strain KT462 utilizes BT and alkyl-substituted derivatives, whereas *R. erythropolis* KA2–5-1 can only attack alkylated forms of (D)BT (Kobayashi *et al.*, 2000; Tanaka *et al.*, 2002). Similarly, *Rhodococcus* sp. strain WU-K2R utilizes DBT and naphtho[2,1-*b*]thiophene (NTH) (Kirimura *et al.*, 2002). Additionally, a problem arises because the metabolites sulfate methionine and cysteine. When present or accumulated due to catabolism, they effectively repress *desulfurization* (*dsz*) gene expression and hence activity (Li *et al.*, 1996). To counteract this, cystathionine synthase (*cbs*) mutants have been produced that enable expression of the *dsz* genes in the presence of high levels of sulfate (Tanaka *et al.*, 2002). An alternative approach has been to clone the *dsz* genes into *Rhodococcus—E. coli* shuttle vectors and then allow expression in *Rhodococcus* using different promoters (Noda *et al.*, 2003; Watanabe *et al.*, 2003). In efforts to further understand

and enhance the process, the desulfinase (DszB) of *Rhodococcus* sp. strain IGTS8 has been structurally characterized (Lee *et al.*, 2004).

Related to the above compounds, some persistent thiocarbamate and *s*-triazine herbicides (De Schrijver and De Mot, 1999) and 2-mercaptobenzothiazole (used in the rubber industry as a vulcanization accelerator) (Haroune *et al.*, 2004) can also be catabolized by *Rhodococcus* strains. Compounds that pose the challenge of being nitro-substituted triazines are particularly interesting. A good example is hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), which is a high explosive that only rhodococci appear to be able to degrade. In *Rhodococcus* strain DN22 this is via an initial denitration reaction followed by ring cleavage to produce formaldehyde and the dead end product 4-nitro-2,4-diazabutanal (NDAB) (Fournier *et al.*, 2002). However, in contrast to this observation, *Rhodococcus rhodochrous* strain 11Y completely degrades RDX with apparently no dead-end metabolites accumulating. In order to achieve this, the initial attack is by a cytochrome P450, then denitration and spontaneous ring cleavage (Seth-Smith *et al.*, 2002).

E. The Role of Oxygenases

Microbial oxygenases are very important enzymes in mediating the direct fixation of oxygen into organic molecules. This is a key step in mediating the recycling carbon in the biosphere. Indeed, it is likely that the success of members of the genus *Rhodococcus* in degrading a wide range of pollutants in the environment is largely based upon the possession of a wide range of oxygenases. For example, the involvement of heme-containing cytochrome P450 enzymes in the degradation of substituted aromatics (Karlson *et al.*, 1993), thiocarbamates, atrazine (Nagy *et al.*, 1995a,b), and ethyl *t*-butyl ether (Chauvaux *et al.*, 2001) has been demonstrated. These enzymes mediate incorporation of a single atom of oxygen into the organic substrate.

In most cases noted to date, the cytochrome P450 redox partners (usually a ferredoxin and reductase) are synthesized as separate polypeptides. However, the cytochrome P450 of *Rhodococcus rhodochrous* strain 11Y has a flavodoxin domain fused at the N terminus (Seth-Smith *et al.*, 2002). This appears to be a ''self-sufficient'' arrangement that has also been observed for the P450 from the camphor degrading *Rhodococcus* sp. strain NCIMB 9784 (Hunter *et al.*, 2005; Roberts *et al.*, 2002, 2003). However, it was noted some time ago that a fatty acid hydroxylase P450 (Gustafsson *et al.*, 2004; Narhi and Fulco, 1986) has a similar arrangement, and other examples have been

observed (De Mot and Parret, 2002) indicating that fusion with a reductase, not normally associated with P450, may have taken place in several cases to yield more efficient enzyme activities. In contrast to P450 oxygenases, aromatic hydrocarbon ring-hydroxylating dioxygenases mediate the fixation of both atoms of molecular oxygen into aromatic compounds such as the high-priority pollutants, polycyclic aromatic hydrocarbons (PAHs). Significantly the reactions that they catalyze are stereospecific, and this means that they can also produce single enantiomers of optically pure compounds as products. The success of members of the genus *Rhodococcus* in degrading aromatic compounds is based upon the possession of a wide range of dioxygenases. The exploitation of *Rhodococcus* oxygenases has been reviewed and illustrates the range of dioxygenases in these bacteria (O'Brien *et al.*, 2002). They belong to the Rieske nonheme oxygenases as defined by Gibson and Parales (2000). The current paradigm regarding dioxygenase function is based upon the *Pseudomonas* naphthalene dioxygenase. However, the discovery of a distantly related naphthalene dioxygenase in *Rhodococcus* sp. strain NCIMB12038 suggests a wider diversity than previously thought (Larkin *et al.*, 1999; Moser and Stahl, 2001). The structure of the terminal component of *Rhodococcus* sp. NCIMB 12038 naphthalene dioxygenase (NarAa, Ab) has been elucidated (Gakhar *et al.*, 2005; Malik *et al.*, 2002), and despite the distant sequence relationship there is considerable structural conservation of the $3\alpha3\beta$ subunit arrangement. Although a key difference appears to be an increased thermal stability of the NarAa, Ab structure, the mechanisms appear likely to be very similar. Interestingly, although it was previously thought that a multicomponent alkene monooxygenase from *Rhodococcus rhodochrous* B-276 may have a very different mechanism to previously characterized methane monoxygenases in other bacteria, recent work has indicated this not to be the case (Fosdike *et al.*, 2005). A similar crystal structure of the terminal oxygenase components (BphA1A2) of the biphenyl dioxygenase from *Rhodococcus* strain sp. RHA1 has also been determined (Furusawa *et al.*, 2004).

There has also been considerable interest in the ring cleavage extradiol dioxygenases that are often associated with the degradation of many aromatic compounds, and sequences of different classes have been reported (Irvine *et al.*, 2000). In *Rhodococcus erythropolis* strain YK2, five extradiol dioxygenase genes (*edi1*, *edi2*, *edi3*, *edi4*, and *dfdB*) have been identified (Iida *et al.*, 2002) with some similarities to the 2,3-dihydroxybiphenyl 1,2-dioxygenases (*bphC1*, *bphC2*, and *bphC3*) from the PCB degrading strain *R. globerulus* P6

(McKay *et al.*, 2003; Vaillancourt *et al.*, 2003). A recent comparative study of the capabilities of mono-, di-, and trichlorinated (triCl) 2,3-dihydroxybiphenyls extradiol dioxygenases in different bacteria indicated that one such enzyme from *Rhodococcus globerulus* P6 had adapted well to the chlorinated substrates (Fortin *et al.*, 2005). It exhibited greater activity against six chlorinated dihydroxybiphenyls than the parent compound, dihydroxybiphenyl, with maximal activity against 4,3′,5′-trichlorodihydroxybiphenyl. *Rhodococcus* strain DK7 catabolizes *o*-xylene and toluene via 3,4-dimethylcatechol and 3- and 4-methylcatechol, which are then attacked by common extradiol dioxygenase (AkbC) that appears to be a new class of this type of enzyme (Kim *et al.*, 2005a). The crystal structure of the 4-chlorocatechol 1,2-dioxygenase from *Rhodococcus opacus (erythropolis)* 1CP, an Fe(III) ion-containing enzyme involved in the aerobic biodegradation of chloroaromatic compounds has a been determined (Ferraroni *et al.*, 2004).

F. Fuel Oxygenates and Ether Bonds

The fuel oxygenates methyl tert-butyl ether (MTBE), ethyl tert-butyl ether (ETBE), and tert-amyl methyl ether (TAME) possess ether bonds and some bacteria, including rhodococci (Goodfellow *et al.*, 2004; Mo *et al.*, 1997) and related actinomycetes (Francois *et al.*, 2002, 2003), have been shown to degrade them. For example, *Rhodococcus ruber* IFP 2001 attacks ETBE using a P450 oxygenase encoded in a defined gene cluster (*ethABCD*) (Chauvaux *et al.*, 2001). Although the mechanism of attack on MTBE by rhodococci has not been similarly elucidated, *Rhodococcus erythropolis* K2–3 has been shown to cleave the ether bond of the phenoxybutyrate herbicides, 4-(2,4-dichlorophenoxy)butyrate (2,4-DB), and 4-(4-chloro-2-methylphenoxy)butyrate (MCPB) using a P450 enzyme (Mertingk *et al.*, 1998; Strauber *et al.*, 2003). Also *Rhodococcus* sp. strain DTB has been shown to utilize bis(1-chloro-2-propyl) ether (DDE) and diethyl ether attacking the ether bond using a flavin-dependent enzyme with dehalogenation occurring at a later stage (Moreno-Horne *et al.*, 2003, 2005). Other *Rhodococcus* strains have been characterized that attack ether bonds and degrade alkyl ethers, aralkyl ethers, and dibenzyl ether (Kim and Engesser, 2004, 2005). It would be of considerable interest to further elucidate if there is a common mechanism involved in the attack on the ether bonds and degradation of this diverse range of compounds.

IV. Environmental Distribution and the Significance of Gene Transfer

Rhodococci have been isolated commonly from soils, waters, and sediments around the world. These have included samples from a high-level nuclear waste plume (Fredrickson *et al.*, 2004) and even samples from a medieval grave (Takeuchi *et al.*, 2002). Although *Rhodococcus* cells can be difficult to lyse and some molecular genetic studies on environmental samples may have failed to detect them (Kauffmann *et al.*, 2004) it is clear that their genes are widely distributed and found in different bacteria in the environment. A hybridization study using a 50-mer-based oligonucleotide microarray of 2402 genes indicated that naphthalene degradation genes from *Rhodococcus* were dominant in naphthalene enrichments but that the catabolic genes from Gram-negative microorganisms, such as *Ralstonia*, *Comamonas*, and *Burkholderia*, were most abundant in soil microcosms (Rhee *et al.*, 2004). Rhodococci have been detected in tropical and artic soils (Luz *et al.*, 2004), and most surprisingly in very deep-sea sediments (Brandao and Bull, 2003; Brandao *et al.*, 2003). Indeed, *Rhodococcus erythropolis* was noted as making up the majority of nitrile-degrading microorganisms from many marine and terrestrial samples (Brandao *et al.*, 2003). It would be therefore of interest to determine if the nitrile biodegradative genes are also conserved in these bacteria since there is mounting evidence that homologous catabolic genes are widely distributed in the rhodococci. Interestingly, it has been noted recently that the nitrile hydratase/amidase gene clusters in *Rhodococcus erythropolis* strains AJ270 and AJ300 and *Microbacterium oxydans* AJ115 isolated from the same area are identical (O'Mahony *et al.*, 2005).

Another clear example that provides evidence of horizontal gene transfer is the complete conservation of the *Rhodococcus rhodochrous* NCIMB13064 halohydrolase gene (*dhaA*) in several strains (Kulakova *et al.*, 1997). Five *Rhodococcus* strains from different sites in Europe, Japan, and the United States share a 12.5-kb region along with shorter regions in *Pseudomonas pavonaceae* 170 (degrading 1,3-dichloropropene) and *Mycobacterium* sp. strain GP1 (degrading 1,2-dibromoethane) (Poelarends *et al.*, 2000a,b). Evidence for the mechanism by which these genes may have been acquired in strains 170 and GP1 is provided by the nearby location of a putative *integrase* gene. Similarly, genes for the alkene monooxygenase (Amo) of *Rhodococcus corallinus* B-276 arose through gene duplication and spread through horizontal gene transfer (Leahy *et al.*, 2003). Homologous biphenyl catabolic (*bph)* genes are found in *Rhodococcus globerulus* P6, *Rhodococus* sp. strain

RHA1, and *Rhodococcus erythropolis* TA421 indicating a common origin in these cases. However, the *bph* genes and gene order in *Rhodococcus rhodochrous* strain K37 indicates that there was probably a different origin (Taguchi *et al.*, 2004). Similarly, the gene order for several catabolic genes in more than one strain including those for naphthalene (*nar*) (Kulakov *et al.*, 2005), alkane (*alkB1-alkB4*) (Whyte *et al.*, 2002), and dibenzofuran (Iida *et al.*, 2002) are conserved.

V. Genetic Basis of Biodegradation Capability

Despite an increased understanding of the wide range of catabolic abilities that rhodococci possess, an understanding of their genetics and recombination mechanisms is still far from advanced. *Rhodococcus* genetic diversity is immense and the selection of a representative strain is difficult. A feature that can influence segregation of genetic elements, and which is often not considered, is their cellular pleomorphism. Many strains grow as short rods and cocci and also produce multinucleated filaments (Locci and Sharples, 1984; Williams *et al.*, 1976). Multinucleated filaments are often only produced in the exponential phase of growth before they fragment to short rods and cocci due to nutrient starvation in the stationary phase (Lefebvre *et al.*, 1978). This can, therefore, lead to problems in the efficient segregation of mutants.

It has been observed, but not formally investigated in any meaningful way, that rhodococci commonly exhibit considerable genomic instabilities that can be either specifically selected or arise spontaneously at numerous loci. Indeed in one of the first authoritative reviews of these bacteria, Waksman in the text *The Actinomycetes* (Waksman, 1950) referred to nocardias (that now encompasses the rhodococci) as being genetically unstable. He concluded that: ''In view of these variations, the question was raised: Is it possible that many of the *Nocardia* species represent degenerate forms of *Streptomyces*?'' Such genetic instability probably lies at the root of these bacteria being able to acquire genes and mediate rearrangements in adapting to many different substrates in the environment.

A. Genome Size and Composition

Consistent with the immense catabolic diversity of rhodococi, it is not surprising that the genome sequence of *Rhodococcus* sp. strain RHA1 is large and reveals 9.7 Mb of genome sequence that is shared between three large linear plasmids; pRHL1 (1100 kb), pRHL2 (450 kb),

and pRHL3 (330 kb) and the chromosome (http://www.bcgsc.bc.ca/cgi-bin/rhodococcus/blast_rha1.pl). There is also a significant degree of gene redundancy. There are numerous oxygenases present including at least six ring-hydroxylating dioxygenases and 10 P450s, and this probably explains why so many strains can adapt to catabolize additional substrates that they were not selected on originally. The large genomes (over 7 Mb each) of two other rhodococci, *Rhodococcus aetherovorans* strain I24 and *Rhodococcus erythropolis* strain PR4, have also indicated the presence of multiple gene homologues. Additionally, it has been noted that multiple alkane hydroxylases may well be a common feature of many *Rhodococcus* strains (van Beilen *et al.*, 2002). Further analysis of these large genomes will no doubt reveal a wealth of catabolic genes and capacity. For example, analyses of the genome of *Rhodococcus* sp. RHA1 indicate gene cluster that putatively encodes genes for taurine-pyruvate aminotransferase (Tpa) and alanine dehydrogenase (Ald) and for the catabolism and regulation of taurine catabolism. Recent experimentation confirms growth on taurine and involvement of these genes (Denger *et al.*, 2004). However, it is notable that they are separated from the associated, sulfoacetaldehyde acetyltransferase (*xsc*), phosphotransacetylase (*pta*), and possible ABC transporter (*tauBC*) genes. The mechanism of coregulation of these genes from different locations remains to be resolved and this issue will be explored further.

B. Catabolic Plasmids

As noted earlier, it is not surprising that many of the genus *Rhodococcus* cells have been also shown to possess a wide variety of plasmid DNA molecules. These range from small, cryptic-closed, circular plasmids to the large, linear plasmids noted earlier. Indeed, virtually every *Rhodococcus* strains analyzed to date appears to harbor such plasmid DNA. Linear plasmid DNA molecules have been detected in many strains of *Rhodococcus* species and *R. fascians* has been shown to possibly possess a linear chromosome of about 900–1000 kbp. The mechanisms of replication or the ends of these molecules have not been characterized in many cases. However, in pHG207 (a recombinant of the wild-type plasmids, pHG204 and pHG205, associated with hydrogen autrophy in *R. opacus*) it was noted that the 5′ ends consisted of imperfect inverted repeats of 176 bp (Kalkus *et al.*, 1990, 1993, 1998) that is typical of actinomycete invertrons.

In RHA1, the biphenyl/PCB degradative (*bph*) genes are distributed around the genome with some genes located on the linear plasmids

(Shimizu *et al.*, 2001). The sequence of pRHL3 has also been shown to be a typical actinomycete invertron, containing large terminal inverted repeats with a tightly associated protein (Warren *et al.*, 2004). Unusually, over 20% of the 300 putative genes are in three catabolic clusters, and four regions appear to have been acquired by recombination. Several insertion sequences, transposase genes and gene duplications are evident. Earlier, there were similar observations for pBD2 from the isopropyl benzene utilizing strain *Rhodococcus erythropolis* BD2 (Stecker *et al.*, 2003). In this case there were 99 putative genes, 23 of which probably have catabolic functions and 32 have possible transposition functions, some of which flank the *ipb* genes. Also, the presence of both *ipb* and *bph* operons indicates that they were probably acquired via transposition and linear plasmid transfer. *Rhodococcus* sp. strain IGTS8, noted earlier, possesses a 150-kb plasmid encoding genes that are involved in the desulfurization of organosulfur compounds (Denis-Larose *et al.*, 1997). Large linear plasmids from rhodococci that encode genes for the catabolism of trichloroethene (Saeki *et al.*, 1999), naphthalene (Kulakov *et al.*, 2005; O'Brien *et al.*, 2002; Uz *et al.*, 2000), toluene (O'Brien *et al.*, 2002; Priefert *et al.*, 2004), alkylbenzene (Kim *et al.*, 2002), biphenyl (Taguchi *et al.*, 2004), chloroaromatic compounds (Konig *et al.*, 2004) have been observed along with the virulence genes in *Rhodococcus fascians* (Vereecke *et al.*, 2002).

It is becoming more evident that multiple recombinations have resulted in a considerable number of homologous genes scattered around the genome that is made up of linear plasmids and a chromosome. It is notable that very few transposable elements have been characterized (Kulakov *et al.*, 1999; Lessard *et al.*, 1999; Nagy *et al.*, 1997), although there are many putative ones present in the genome sequences. These bacteria also appear to possess the mechanisms for a high frequency of illegitimate recombination (Kulakov and Larkin, 2002; Larkin *et al.*, 1998), and this may mediate, alongside homologous recombination events, the introregression of DNA without the need for many of mobile genetic elements as suggested for *Acinetobacter* (de Vries and Wackernagel, 2002).

C. Genetic Adaptation, Gene Transfer, and Recombination

Interest in the recombination mechanisms of rhodococci stretches back to the pioneering work of Adams in the 1960s. Since then a picture of the recombinational strategies adopted by these bacteria has emerged. As early as 1963, a recombination system that appeared

to be self-incompatible was demonstrated in *Rhodococcus erythropolis* (formally *Nocardia erythropolis* and *Nocardia canicruria*) (Adams, 1964; Adams and Bradley, 1963). This indicated that there may be a novel system of heterothallism whereby recombinations resulting from crosses of different strains were favored and suggested that that extensive DNA homology may not be a significant factor in recombinations. Indeed, in *Nocardia asteroids*, efficient recombination was observed between different strains with overall DNA homology as low as 33% (Kasweck and Little, 1982) and several different mating types have been postulated (Brownell and Adams, 1968; Brownell and Kelly, 1969). The system of several mating type loci previously postulated to be controlling this phenomenon appears, in part at least, to be associated with an 8-kb segment of DNA located on the 60–80-kb plasmid pDA20 and its 15-kb derivative pDA21 (Gowan and Dabbs, 1994). These mechanisms have yet to be elucidated further and it is possible that the loci which control mating types also determine the outcome and frequency of recombination. The ability of different strains to recombine readily indicates the remarkable flexibility of the *Rhodococcus* genome.

Although the overall DNA homology between compatible strains of *Rhodococcus* can appear to be surprisingly low (Kasweck and Little, 1982) it is also clear that there are regions of close homology. For example, when DNA from *R. erythropolis* mating type cE2 is reassociated with DNA from the compatible mating type Ce3, there was only approximately 60% homology evident. Yet when the reassociation was carried out in reverse almost 100% homology was observed. This indicates that the Ce3 strain had a considerable amount of additional nonhomologous DNA and can now be easily explained by the probable large linear plasmids as described earlier.

The flexibility of the *Rhodococcus* genome with regard to recombination is further demonstrated in studies of plasmid integration in *Rhodococcus fascians* (Desomer *et al.*, 1991). Upon electroporation of cells with exogenous DNA, illegitimate integrations appeared to be strongly favored at a short palindromic sequence (CCGCGG) that is conveniently present in one copy of a pUC13 derivative (pRF41) and in two copies in a related plasmid (pRF32). Plasmid insertion into the genome appeared to occur at random, nonhomologous sequences leading to a wide range of mutant phenotypes. The nature of the plasmid constructs indicates that the gene(s) for the site-specific mechanism of integration that acts upon the plasmid DNA are probably host-encoded by the *Rhodococcus.* Therefore it is also likely that exogenous DNA bearing such a sequence would lead to a nonhomologous

integration at a recombinational ''hot-spot'' sequence. Although this phenomenon has not been widely reported or studied, it is possible that rhodococci can use such mechanisms to adapt by recombining easily with a wide variety of heterologous sequences.

D. Gene Regulation

Although the regulation of few *Rhodococcus* biodegradation gene clusters has been investigated, both positive regulators (Komeda *et al.*, 1996) and repressors have been noted (Barnes *et al.*, 1997; Nga *et al.*, 2004). However, there are indications that there may be coregulation of genetically unlinked transcriptional units and this is more intriguing. For example, the multiple biphenyl/PCB degradation genes of RHA1 and M5 are distributed in several clusters (Kitagawa *et al.*, 2001; Yamada *et al.*, 1998) that are located on linear plasmids pRHL1 and pRHl2 (Shimizu *et al.*, 2001). It has been demonstrated that a two-component regulatory system (*bpdST*) regulates expression of some of the *bph* genes in M5 (Labbe *et al.*, 1997) and RHA1 (Takeda *et al.*, 2004a,b) and expression of in the *o*-xylene catabolic genes in *Rhodococcus* sp. strain DK17 (Kim *et al.*, 2005b). However, the regulatory network that must be operating has not been resolved. In contrast to Gram-negative bacteria where LysR regulators have been implicated, a putative GntR-like transcriptional regulation (*narR1* and *narR*2) appears to be involved in several naphthalene degrading *Rhodococcus* strains (Kulakov *et al.*, 2005). Again, in this case there is clear evidence that the catabolic genes are not organized into a single cluster and different strains have several homologous transcriptional units separated by nonhomologous sequences containing direct and inverted repeats. A similar arrangement has been observed elsewhere (Dong *et al.*, 2004). In the case of the naphthalene dioxygenase enzyme complex, the genes for expected ferredoxin and reductase components are not evident and only genes encoding that catalytic components of naphthalene dioxygenase (*narAa,Ab*) and *narB* gene (encoding the naphthalene *cis*-diol dehydrogenase) are transcribed as a single unit upon induction with naphthalene. This implies that there is likely to be coregulation of other catabolic genes, and transcriptional induction of an unlinked *extradiol dioxygenase* genes by naphthalene was demonstrated (Kulakov *et al.*, 2005). This is further complicated by the demonstration of different promoter sequences initiating the expression of the homologous *narAa–narB* gene clusters and suggests that recombination events may be involved in the acquisition and alignment of regulatory regions with the catabolic genes. Similarly, in

Rhodococcus RHA1 noted earlier there is dispersal of the degradation genes for benzoate (*ben*), phthalate (*pad*), uptake of phthalate (*pat*), and the genes for two branches of the β-ketoadipate pathway (*cat* and *pca*). Indeed some are contained on a putative ''catabolic island'' that is duplicated on plasmids pRHL1 and pRHL2. The regulatory interrelationship between the gene clusters is complex with involvement of *pad* and *pat* gene products in phthalate degradation and *ben* and *cat* gene products in benzoate degradation. Expression of the *pca* products is also implicated as they are present after growth on both substrates (Patrauchan *et al.*, 2005). Further analysis of the *Rhodococcus* RHA1 genome indicates that utilization of phenylacetic acid degradation is encoded in part by 13 *paa* genes on chromosome. A single transcript encodes 11 genes but production of a further 146 proteins was induced by growth on phenylacetic acid (Navarro-Llorens *et al.*, 2005).

VI. Conclusions and Prospects

Analysis of the biochemical diversity physiology and genetics of many *Rhodococcus* strains is revealing why they can adapt to catabolize many different substrates. Whole genome analysis indicates that they have acquired many copies of genes in very large genomes that include large linear plasmids. Associated with this appears to be a relatively flexible recombinational system that underlies a hyper-recombination evolutionary strategy. The storage of many genes enables them to be deployed as recombination substrates upon adaptation to new substrates. It is likely that bacteria adopt different genetic strategies to generate variation that is consistent with the environment that they are found in. Other bacteria, such as the *Mycoplasma* spp. (Rocha and Blanchard, 2002), *Helicobacteria pylori* (Salaun *et al.*, 2004), and *Rickettsia* spp. (Andersson *et al.*, 1998; McLeod *et al.*, 2004) have much smaller genomes and lack DNA recombination and repair functions. On the other hand, a commensal and pathogenic bacterium, such as *Bacteroides fragilis* (Patrick *et al.*, 2003), mediates antigenic variation through numerous gene switches at specific loci. Rhodococci, however, inhabit environments that contain many possible carbon and energy sources. Consistent with this, their genomes are large and possess mega linear plasmids that act as storage for multiple copies of many biodegradative genes. There is also evidence that catabolic genes are coregulated in separate gene clusters arising from multiple adaptive recombinations. It would be interesting to investigate further this regulation and determine how the genes are arranged in the genome with respect to each other. It has been postulated (Rocha and

Danchin, 2003) that essential genes tend to be more conserved in the leading strand due to selection. However, this may not be the case for the gene arrangement in *Rhodococcus.* It is likely that the capabilities of these bacteria with respect to the catabolism of many organic compounds will continue to be exploited in remediation of pollutants in the environment. Additionally, investigation of the many and diverse genes that they possess will lead to their continued exploitation in commercial biotransformation.

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Genomes as Resources for Biocatalysis

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I. Introduction

How well biocatalysis enables novel routes to target molecules ultimately rests on the properties of the available enzymes. In most applications, proteins are called on to catalyze reactions for which they did not specifically evolve. This means one must first identify the best enzyme ''tool'' for a particular synthetic task, and this phase of bioprocess development has often consumed a significant fraction of the total effort expended. Biocatalyst identification was originally conducted by screening whole organisms (usually bacteria and fungi) to uncover those that catalyzed the desired transformation. This strategy has proven especially successful with steroid biotransformations in which site-selective hydroxylation or carbonyl reductions are the typical goals (Mahato and Majumdar, 1993; Warhurst and Fewson, 1994). Many organizations have accumulated large microbial culture collections, and these have provided the ''hits'' that have been—and continue to be—developed into commercial bioprocesses.

Despite the many success stories derived from whole organism screening, this approach suffers from several drawbacks. First, the strategy is purely empirical, and the screening process must be repeated for each new substrate/reaction pair. While accumulated experience can help narrow the search to those organisms known to mediate related reactions, the exploration remains labor-intensive since cells of each organism must be grown anew for every screening. Another problem with using whole cells is that they often catalyze side-reactions in addition to the desired conversion. The enzyme of interest rarely makes up more than a few percent of the total protein in a native

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 59 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(06)59002-1

microbial cell, and its activity level is often insufficient to outcompete other enzymes for access to the substrate. The reaction product may also be subject to degradation by the organism. The traditional solution to these problems of low activity and overmetabolism was to identify additional microorganisms that share the same (or closely related) enzyme of interest but with a different complement of competitors and/or expression level. The high degree of horizontal gene transfer among bacteria makes this approach particularly valuable in these organisms.

Access to the enzyme of interest in pure form side-steps the problems outlined earlier. Until recently, however, few synthetically useful enzymes were commercially available. Lipases and other hydrolases were the first class for which a variety of enzymes were packaged into kits that could be screened by bench chemists with no particular biological expertise. It is no accident that hydrolase applications blossomed after this development. Other enzyme classes, such as dehydrogenases, have been similarly targeted for expansion (Zhu *et al.*, 2005). Commercial enzymes are nearly always produced by recombinant systems, and these engineered cells can also be used directly as the biocatalytic reagent since the enzyme of interest often makes up $\geq$20% of total cellular protein. At such elevated specific activity levels, competition with other enzymes and product overmetabolism are often insignificant. Moreover, for those enzymes that require cofactors (e.g., dehydrogenases) intact cells allow regeneration by their native metabolic pathways.

Cloned enzymes for biocatalysis have traditionally been obtained by first isolating the protein from a strain identified as a good ''hit'' in an organism-screening program. Amino acid sequence data were then used to design primers for initial cloning and sequencing, and then the gene was overexpressed in a heterologous host (for an example of this strategy, see Costello *et al.*, 2000). While this approach has been highly successful and remains useful, it can be time-consuming because the enzyme must initially be purified from an organism in which low levels are typically present. In addition, the DNA of some organisms, such as those with high-GC content and multiple, closely related genes, can pose difficulties in the cloning steps.

The advent of routine genome sequencing has dramatically expanded our inventory of putative protein sequences, which has now made a reverse-genetics approach to biocatalyst discovery feasible. This strategy uses bioinformatics methods to identify genes that may encode enzymes useful for synthetic transformations. These are then expressed, purified, and evaluated for their suitability. The key

advantage of reverse-genetics is that it eliminates the need for painstaking purification of low-abundance proteins prior to gene cloning; instead, the gene itself becomes the starting point for an expedited screening process. The power of the method increases in direct proportion to the number of sequenced genomes and our accuracy in assigning functions to the hypothetical proteins.

This chapter describes our efforts to use a reverse-genetics approach to building a library of synthetically useful dehydrogenases from the genome of bakers' yeast (*Saccharomyces cerevisiae*). This pairing of reaction type and organism was deliberate. There is a nearly 100-year history of employing whole yeast cells to reduce a broad array of aldehydes and ketones (D'Arrigo *et al.*, 1997; Nakamura *et al.*, 2003; Neuberg, 1949; Santaniello *et al.*, 2000). It is arguably the only living organism that is accepted as a ''normal'' reagent in the organic laboratory because of its combination of low cost, lack of toxicity, and ready availability. It was also the first genome to be completely sequenced (Goffeau *et al.*, 1996). Finally, while the range of acceptable substrates is large, the stereoselectivities of whole-cell bioconversions are often modest, despite hints that individual yeast dehydrogenases might be highly selective (Katz *et al.*, 2003; Nakamura *et al.*, 1991; Shieh *et al.*, 1985). Because only a handful of yeast reductases had been identified by the traditional purification/gene cloning route, we selected this as an ideal system for proof-of-principle. The methods are general, however, and can be applied to any genome and reaction type of interest.

II. Yeast Dehydrogenase Gene Identification

The details of methods used to identify dehydrogenases encoded by the *S. cerevisiae* genome have been described in detail by Stewart *et al.* (2001), and only a brief overview is provided here. Biochemical studies of proteins from a wide variety of sources have revealed several classes of dehydrogenases, including aldose reductases, D-hydroxyacid dehydrogenases, and medium- and short-chain dehydrogenases. We, therefore, used protein sequences of known dehydrogenases as ''probes'' to analyze the *S. cerevisiae* genome and identify known and hypothetical genes with similar sequences. This process was repeated for each superfamily and resulted in a list of sequences that might correspond to actual dehydrogenase enzymes. Because each dehydrogenase superfamily is marked by specific amino acid sequence motifs, genes lacking these patterns were excluded, as were sequences not possessing residues known to be involved in catalysis. The final acceptance criteria

were based on structure. The sequence of each potential yeast dehydrogenase was aligned with a superfamily member whose three-dimensional structure had been determined by X-ray crystallography. Those with insertions or deletions within elements of secondary structure were discarded, since these make it unlikely that the yeast sequence could achieve a similar fold. Finally, the locations of insertions or deletions were mapped onto the known crystal structures and examined manually. In all cases, the changes could be reasonably accommodated by changes in surface loops, leaving the core intact. The 49 yeast sequences that passed all of these tests were retained as potentially useful dehydrogenases.

We concentrated our initial attention on the 23 yeast open reading frames (ORFs) considered most likely to be useful in synthesis. Some proteins were eliminated because they were already available commercially (yeast alcohol dehydrogenase and formate dehydrogenase) or known to have very limited substrate acceptance (other yeast alcohol dehydrogenases, lactate dehydrogenases). Finally, we also deferred study of several putative proteins that were only weakly similar to authentic short-chain dehydrogenases. Of the remaining sequences, the catalytic properties of only four had been examined previously in any detail (those encoded by the *YOR120w, YHR104w, YDR368w*, and *YOL151w* ORFs).

One key limitation of the reverse-genetics approach is that the sequences of one or more ''probe'' sequences from proteins with the desired catalytic activity must be available. While this does not pose a problem for well-characterized reaction classes, such as ester and amide hydrolysis, carbonyl reductions, and so on, it may prove a limitation as biocatalysis is extended to more exotic reaction types. Access to genomic DNA and cDNA could also limit the practical utility of the approach. Fortunately, strain collection curators have now made genomic DNA samples available for purchase, removing the need for individual researchers to grow and isolate this material. This is particularly valuable for genes derived from fastidious and/or pathogenic organisms.

III. Expression and Isolation of Yeast Dehydrogenases

Martzen *et al.* (1999) constructed a complete *S. cerevisiae* ORF overexpression library in recombinant yeast cells using a glutathione *S*-transferase (GST) purification tag. This allows a simple, universal purification strategy and maximizes the chances of isolating functional assemblies from individual cloned ORFs. Unfortunately, these

S. cerevisiae strains afforded only modest yields of the fusion proteins. While these levels were sufficient for identifying the native reactions catalyzed by yeast enzymes, we were unable to identify unnatural substrates for dehydrogenases reproducibly using this system.

Overexpressing the same GST-fusion proteins in *Escherichia coli* solved most of the dehydrogenase availability issues. We used a standard T7 RNA polymerase/promoter system, and typical overexpression levels were 10–20% of total cellular proteins. Pure GST-fusion proteins were isolated from crude cell extracts after chromatography on commercial glutathione-containing resin (Kaluzna *et al.*, 2004).

Of the 23 yeast ORFs originally targeted for *E. coli* expression, we failed to isolate useful quantities of fusion proteins from four (YCR105w, YJR159w, YMR318c, and YOR388c). The first three belong to the medium-chain dehydrogenase superfamily and are predicted to bind one or more essential Zn^{2+} ions. We did not attempt to improve protein expression levels by supplementing the growth medium with Zn^{2+} or by employing other strategies designed to avoid inclusion body formation. Our experience has been that screening typically reveals several dehydrogenases with the desired stereoselectivity toward a substrate. Devoting inordinate time and effort to correct expression problems of a single enzyme is therefore not usually worthwhile. Instead, we believe that expanding the collection of clones should take precedence since this is more likely to yield a well-behaved dehydrogenase with the desired selectivity. Good protein overexpression in *E. coli* becomes a critical parameter for bioconversions destined for scale-up.

IV. Characterization of Yeast Dehydrogenases

The most useful catalysts for asymmetric organic synthesis—either chemical or biological—possess both broad substrate acceptance and high stereoselectivities. With a few exceptions, the properties of the yeast proteins in our collection were unknown. In addition, we had targeted several ORFs that were only hypothetical proteins, and it was essential to demonstrate that these were both stable and functional dehydrogenases. We addressed these issues by examining a homologous series of β-keto esters (Fig. 1) (Kaluzna *et al.*, 2004, 2005). These ketones were chosen because the alcohol products are useful synthetic intermediates, whole yeast cells have used to reduce similar compounds (albeit with poor stereoselectivity in many cases) and the substituents allow both steric and electronic effects to be probed (Sybesma *et al.*, 1998). β-Keto esters that lack α-substituents (R_2 = H: **1a, 1f**, and **1i**) can give only enantiomeric alcohols; the others can potentially

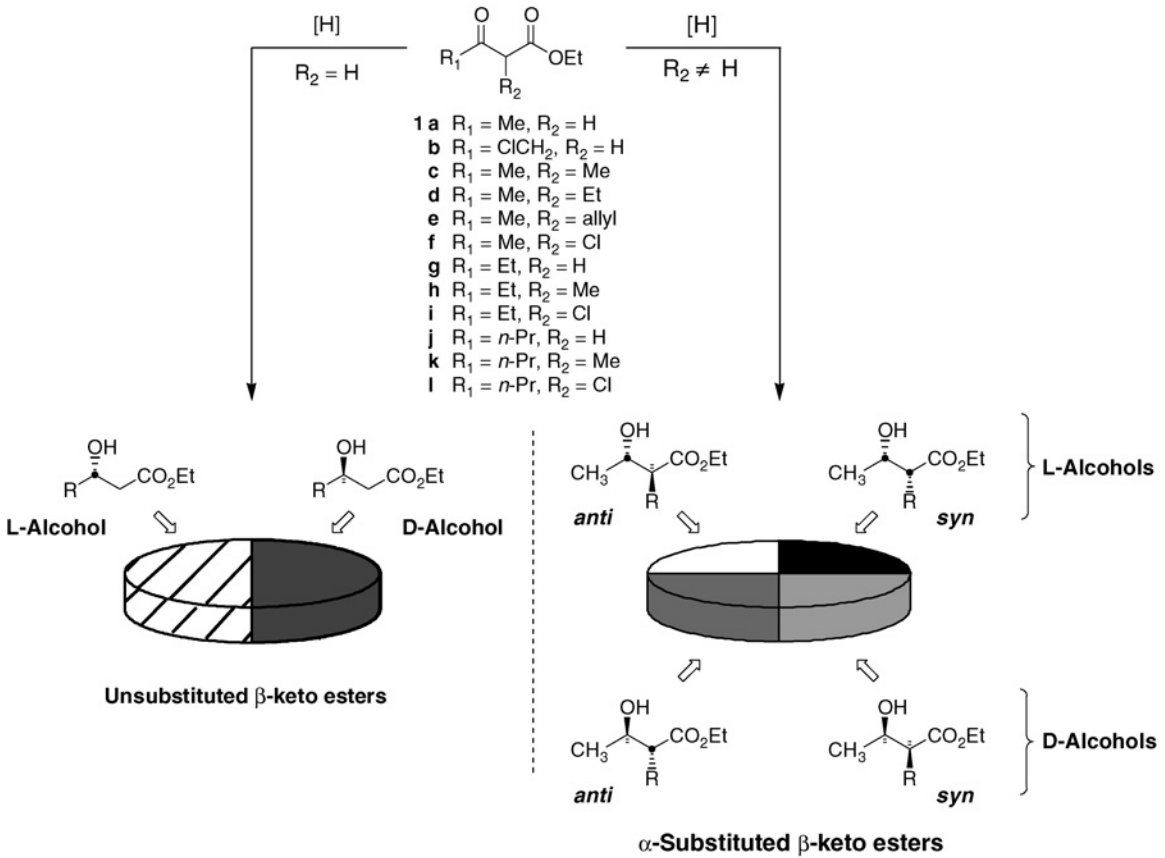


FIG. 1. β-Keto ester sustrates and products.

afford four products. The substrates can also be divided into ethyl acetoacetates and higher homologs. This grouping proved useful in identifying patterns of substrate acceptance and stereoselectivities.

A. Results from Ethyl Acetoacetates

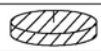
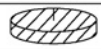
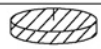
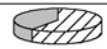
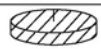
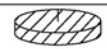
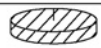
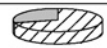
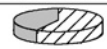
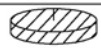
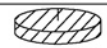
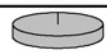
We examined the reductions of six ethyl acetoacetates by 18 purified yeast dehydrogenases, expressed as GST-fusion proteins. The results are collected in Table I, using pie charts to indicate the product compositions of reactions that proceeded to $\geq$20% completion after 24 hours. This degree of fractional conversion was chosen to highlight those reactions with greatest potential synthetic utility; the product quantity could be increased by higher enzyme concentrations, if desired.

Several points are apparent from the collected data. First, virtually every reduction of an ethyl acetoacetate by every yeast dehydrogenase proceeded with complete L-selectivity. The minor amount of D-alcohol produced by the YNL331c protein from ethyl acetoacetate itself and several reductions of **1b** were the only exceptions. This stereochemical convergence is remarkable, given the wide differences in protein and substrate structures within the collection. Is the L-selectivity related to the stereochemical demands of the enzymes' normal metabolic roles? While this is a tempting speculation, the problem is that the ''natural'' substrates for these dehydrogenases are unknown. Five- and six-carbon ketoses are strong candidates, however. The fact that *S. cerevisiae* responds to osmotic stress by producing sugar alcohols, such as sorbitol, and genes encoding nearly half the dehydrogenases studied here are upregulated greater than or equal to twofold under these conditions (Causton *et al.*, 2001)[1] makes this suggestion at least plausible. In addition, several yeast enzymes in our collection accept carbohydrates as substrates. Träff *et al.* (2002) demonstrated that proteins encoded by the *YJR096w, YOR120w, YHR104w*, and *YDR368w* genes can reduce xylose and arabinose. The *YBR149w* gene encodes a subunit of arabinose dehydrogenase (Kim *et al.*, 1998), and the medium-chain dehydrogenase product of the *YLR070c* gene is known to reduce xylitol (Richard *et al.*, 1999). Whether these carbohydrates are the sole, or even optimal substrates, for these enzymes is not known, however.

[1] Genes upregulated $\geq$ twofold by saline osmotic stress: *YJR096w*, *YBR149w*, *YOR120w*, *YHR104w*, *YDR368w*, *YNL274c*, *YGL157w*, and *YOL151w* (Causton *et al.*, 2001).

TABLE I

BIOCATALYTIC REDUCTIONS OF ETHYL ACETOACETATES*

Yeast gene	1a	1b	1c	1d	1e	1f
YJR096w			---†	---	---	
YDL124w			---		---	
YBR149w					---	
YOR120w						
YCR107w				---		
YNL331c			---		---	
YHR104w	---		---	---	---	
YDR368w						
YGL185c	---		---	---	---	
YNL274c	---			---	---	
YP*L275w*				---	---	
YPL113c				---		
YLR070c			---	---	---	
YAL060w						
YGL157w						
YDR541c						
YGL039w						
YOL151w						
Yeast cells						*b*

*Yeast enzymes are referred to by their genetic codes. Product compositions from reactions that proceeded to ≥20% conversion within 24 hours are shown in pie charts (legend in Fig. 1).

†<20% conversion after 24 hours.

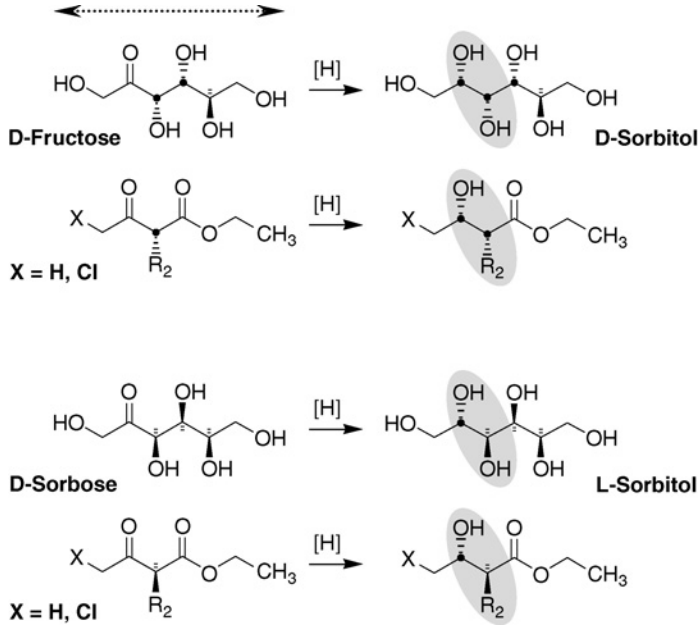


FIG. 2. Potential correspondence between 2-ketoses and β-keto ester substrates.

All common ketohexoses have carbonyl groups located at C2, and their overall lengths are similar to those of ethyl acetoacetates (Fig. 2). A dehydrogenase binding site optimized for 2-ketohexoses could also reasonably accommodate a β-keto ester, and analogous binding would position the β-keto ester ketone carbonyl in a location and orientation conducive to hydride transfer. This hypothetical binding arrangement also allows the β-keto ester C1 carbonyl to interact with protein moieties that ordinarily accommodate the polar C4 hydroxyl of a ketohexose.[2] This arrangement suggests that chlorine-substituted ethyl acetoacetates **1b** and **1f** are excellent substrates for all the dehydrogenases examined here because the electronegative Cl moieties may contact protein residues that ordinarily interact with a ketose hydroxyl. On the other hand, a binding subsite optimized for a ketohexose C3

[2] The D-configuration at C4 shown in Scheme 2 was selected because D-sugars are much more common than their L-counterparts. The configuration at C3 was chosen to favor the more common ketohexose (D-fructose over D-tagatose; D-sorbose rather than D-psicose). It should be noted that these configurations also correspond to the ''glucose'' pattern.

hydroxyl moiety may be unable to accommodate a larger substituent, and this may be the reason that only some of the yeast dehydrogenases accept ethyl acetoacetates with bulky α-substituents. Finally, if the yeast dehydrogenases normally reduce common 2-ketohexoses, such as D-fructose and D-sorbose, the overwhelming L-selectivity in hydride addition to ethyl acetoacetates is simply a consequence of the enzymes' need to recognize substrates with the common ''glucose'' hydroxyl configuration at the ketone carbonyl undergoing reduction. Altering this enantioselectivity would require that the β-keto ester bind in the opposite orientation, which may be difficult for these small substrates whose dimensions are so similar to those of 2-ketohexoses (although this is clearly possible for **1b**). It should be emphasized that the earlier explanation is conjectural, and demonstrating that these enzymes actually reduce ketoses, such as D-fructose and D-sorbose, awaits experimental verification. A similar argument could also be developed for aldoses.

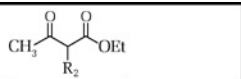
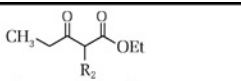
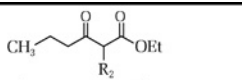
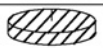
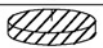
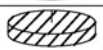
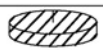
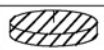
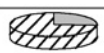
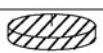
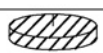
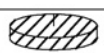
For maximum utility in asymmetric synthesis, a dehydrogenase library should provide all possible alcohol stereoisomers in optically pure form. This goal is only partially met for alcohols derived from **1a–f**. As noted earlier, nearly all of the reductions proceed with very high L-selectivities, and this essentially the only outcome for **1a** and **1c–f**. In cases in which diastereoselectivity is also important (**1c–f**), it is possible in all cases to prepare the L-*syn* diastereomer in homochiral form. By contrast, only the L-*anti* alcohols from **1e** and **1f** can be obtained in >98% ee and 95% de; other reductions proceed with less-than-desirable diastereoselectivities. These shortcomings, as well as the need for D-selective reductases, need to be remedied in an expanded dehydrogenase library.

B. Results from Higher Homologs

In addition to ethyl acetoacetates, we also examined the interaction of higher β-keto ester homologs with the collection of yeast dehydrogenases (Kaluzna *et al.*, 2004, 2005; Stowe and Stewart, 2005). These data, collected in Table II, further illustrate several of the trends noted earlier. Most notably, as the length of the R_1 moiety increases, the fraction of dehydrogenases that reduce a given β-keto ester decreases, unless the molecule contains a chlorine at the α-position (**1i, 1l**). The short-chain dehydrogenases prove the exception to this generalization. These enzymes accommodate larger substrates without apparent difficulty and also carry out reductions with high stereoselectivities for the L-*anti* alcohols. These were also the only enzymes that accepted

TABLE II

COMPARISON OF YEAST REDUCTASE BEHAVIOR TOWARD β-KETO ESTERS WITH VARIATIONS IN α-SUBSTITUENTS*

Yeast gene									
	1a	**1c**	**1f**	**1g**	**1h**	**1i**	**1j**	**1k**	**1l**
YJR096w		---		---	---		---	---	---
YDL124w		---		---	---		---	---	---
YBR149w				---	---		---	---	
YOR120w					---			---	---
YCR107w					---		---	---	
YNL331c		---		---	---			---	
YHR104w	---	---		---	---	---	---	---	---
YDR368w					---			---	
YGL185c	---	---		---	---		---	---	---
YNL274c	---			---	---		---	---	---
YP*L275w*				---	---	---	---	---	---
YPL113c					---			---	---
YLR070c		---		---	---	---	---	---	---

(*continued*)

TABLE II (*Continued*)

YAL060w					---			---	
YGL157w									
YDR541c					---				---
YGL039w									
YOL151w					---	---		---	---

*Yeast enzymes are referred to by their genetic codes. Product compositions from reactions that proceeded to ≥20% conversion within 24 hours are shown in pie charts (legend in Fig. 1).

α-methyl-β-keto esters **1h** and **1k**. Surprisingly, the same enzymes reduced α-substituted ethyl acetoacetates with relatively poor discrimination (Table I), which emphasizes the influence of substrate size on stereoselectivity.

For a few dehydrogenases (YOR120w, YDR368w, and YAL060w), the outcomes of reactions with **1g–l** followed the patterns established by ethyl acetoacetates. In other cases, however, some reductions yielded substantial amounts of product types not observed previously. For example, YJR096w, YDL124w, YGL185c, and YNL274c all produced substantial amounts of D-*anti* alcohol from **1i**; no D-alcohols were observed from their reductions of **1a–f**. This reversal of stereoselectivity underscores the importance of determining reaction outcomes empirically, rather than attempting to generalize using data from related structures.

C. Synthetic Applications

One of the major reasons for investigating enzymatic reductions of α-chloro-β-keto esters was to generate alcohol products that could be employed in total synthesis projects. In particular, chlorohydrins can be cyclized under basic conditions to yield α,β-epoxy esters, also known as glycidic esters. A weak base allows the reaction to proceed with inversion of stereochemistry at C2, so that a *syn*-chlorohydrin yields a *cis*-epoxide (Cabon *et al.*, 1995; Wuts *et al.*, 2000). The densely packed functionality of glycidic esters allows a myriad of subsequent synthetic transformations, and they have become highly valuable intermediates (Adger *et al.*, 1997; Akita *et al.*, 1990; Chong and Sharpless, 1985; Corey *et al.*, 1992; Kato *et al.*, 1997; Matsuura *et al.*, 1994; Schwartz *et al.*, 1992; Smith, 1984; Wuts *et al.*, 2000; Yamada *et al.*, 1998). The chemoenzymatic approaches described later demonstrates both the synthesis and applications of these compounds.

1. *Taxol® Side-Chain Antipodes*

Taxol® (Paclitaxel) has emerged as the drug of choice for several breast and ovarian cancers, and it has become the largest-selling drug in this therapeutic area (Fig. 3) (Morrissey, 2003). The drug's complex structure and limited availability from natural sources has required a semisynthetic strategy for its commercial production (Denis *et al.*, 1986; Holton, 1989). The terpene core, isolated efficiently from the needles of an ornamental shrub, is coupled with the phenylisoserine side-chain prepared by chemical synthesis. Because of its

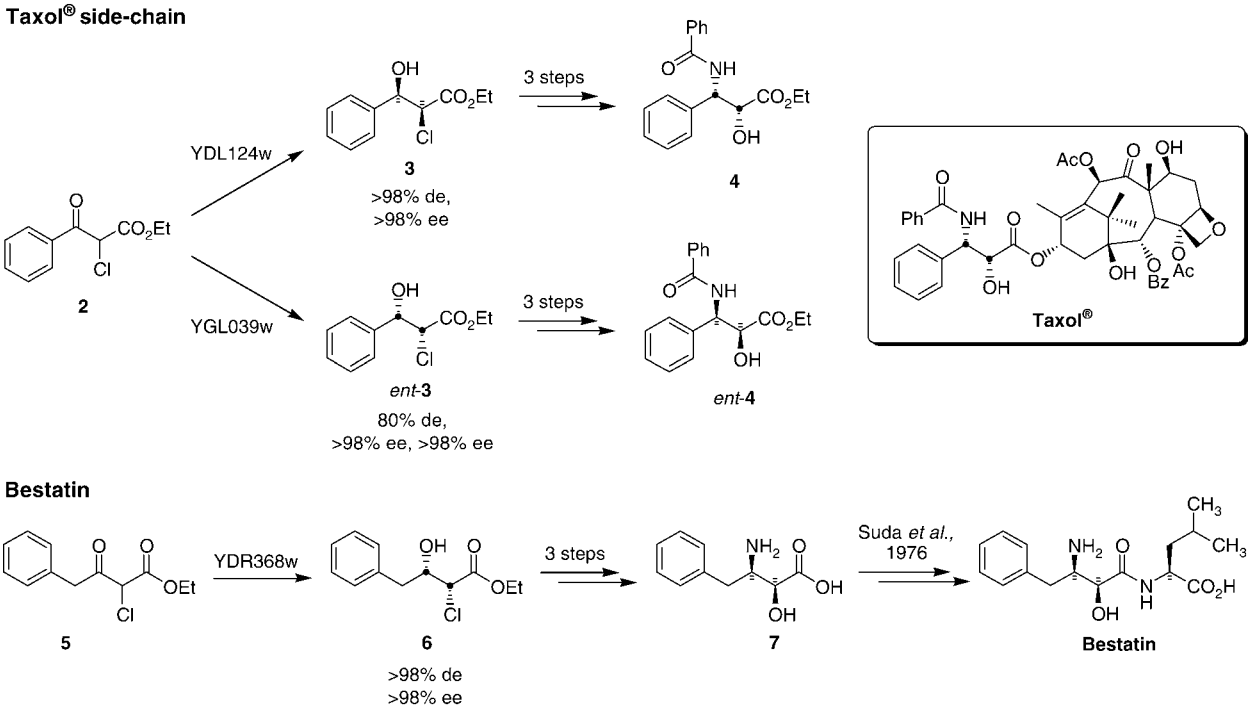


FIG. 3. Chemo-enzymatic routes to α-hydroxy-β-amino acid subunits of Taxol® and bestatin.

economic importance and small size, the Taxol® side-chain has been a popular target for total synthesis, particularly among chemists engaged in methodology development (Borah *et al.*, 2004; Cardillo *et al.*, 1998; Castagnolo *et al.*, 2004; Deng and Jacobsen, 1992; Hamamoto *et al.*, 2000; Lee and Kim, 1998; Lee *et al.*, 1998; Mandai *et al.*, 2002; Srivastava *et al.*, 1994; Voronkov *et al.*, 2003; Wang *et al.*, 1994; Wuts, 1998; Zhou and Mei, 2003). Our goals in preparing **4** were to highlight the synthetic utility of yeast reductases and to develop a more concise route from the *cis*-glycidic ester derived from **3** to the final target.

Screening our collection of individual *S. cerevisiae* dehydrogenases showed that several accepted α-chloro-β-keto ester **2** (Kaluzna *et al.*, 2005). Previous attempts to reduce **2** with whole yeast cells produced a mixture of all four possible alcohol diastereomers (Cabon *et al.*, 1992, 1995). By contrast, aldose reductase YDL124w yielded D-*syn*-alcohol **3** as the only detectable product. This behavior was not completely unexpected, given the results obtained earlier for **1i** with this enzyme (Table II). It should be noted, however, that YDL124w failed to reduce **1l** to a significant extent, further reinforcing the importance of empirical screening in defining the substrate range of a dehydrogenase. Once the optimal dehydrogenase had been identified, preparative-scale reactions were carried out with whole cells of an *E. coli* strain that overexpressed the corresponding GST-fusion protein (Feske *et al.*, 2005). Glucose was added to a cells suspended in phosphate buffer lacking a reduced nitrogen source. Under these conditions, glucose was metabolized rapidly to provide the essential NADPH cofactor; however, the cells were unable to divide and thereby enter stationary phase. Our previous study had shown that these conditions are very useful for conducting NADPH-dependent reactions (Walton and Stewart, 2004), and we were able to isolate >1 g of **3** from a **1l** fermentation. The Taxol® side-chain ethyl ester was then prepared by a three-step sequence that featured a Ritter reaction with benzonitrile to open the *cis*-glycidic ester derived from **3**. This yielded a *trans*-oxazoline intermediate that was hydrolyzed to the final target **4** under acidic conditions (Kingston, 2001).

One benefit to having a library of dehydrogenases is that multiple stereoisomers may be available from a common starting material. This proved to be the case in the reduction of **2**. Short-chain dehydrogenase YGL039w provided the enantiomer of the product obtained from aldose reductase YDL124w. The ability to prepare *ent*-**3** provided the opportunity to synthesize the antipode of the natural Taxol® side-chain. As earlier, preparative-scale reactions were carried out under non-growing conditions with engineered *E. coli* cells. Chlorohydrin *ent*-**3**

was converted to *ent*-**4** by the same route as described earlier. The formation of L-*syn* alcohol *ent*-**3** as the major reduction product by YGL039w would not have been predicted from the data in Table II. Data from the closest analogs, **1i** and **1l**, would have instead suggested that the L-*anti* alcohol should have been the major product. In fact, this diastereomer made up only 10% of the total product with the remainder being the L-*syn* product *ent*-**3**. Experiences, such as this, have made us wary of attempting to formulate general rules for predicting the stereochemical course of enzymatic reductions, since these depend on a complex interplay between the enzyme and substrate.

2. *Bestatin*

The dipeptide bestatin is used clinically in Japan, both for its antibacterial and anticancer activities (Fig. 3) (Abe *et al.*, 2001; Umezawa *et al.*, 1976). Like the Taxol® side-chain, bestatin has been a popular target for total synthesis (Bergmeier and Stanchina, 1999; Koseki *et al.*, 1996; Kudyba *et al.*, 2003; Lee *et al.*, 2003; Nishizawa *et al.*, 1983; Norman and Morris, 1992; Palomo *et al.*, 1994; Pearson and Hines, 1989; Seki and Nakao, 1999; Semple *et al.*, 2000; Suda *et al.*, 1976; Wasserman *et al.*, 2003). *Syn*-α-hydroxy-β-amino acid **7** is the key substructure, and Umezawa has shown that this can be converted to the final target by three routine steps (Suda *et al.*, 1976). Our approach to **7** was similar to that used in the Taxol® side-chain synthesis. In this case, aldose reductase YDR368w was the best dehydrogenase, providing L-*syn* alcohol **6** as the only isolable product (Feske and Stewart, 2005). Here, the configuration of **6** followed from the precedents of **1i** and **1l**. Gram-scale reductions of **5** were carried out with whole cells of the *E. coli* overexpression strain using glucose fed-batch conditions to provide NADPH regeneration. After extraction and purification by column chromatography, chlorohydrin **6** was converted to a *cis*-epoxide that was opened in a Ritter reaction with benzonitrile. Subsequent hydrolysis provided **7** whose spectral data and optical rotation matched values of an authentic standard.

V. Conclusions and Future Directions

In the early yeast dehydrogenase literature, enzymes were referred to as L- and D-selective (Nakamura *et al.*, 1991; Shieh *et al.*, 1985). While this nomenclature was acceptable when only a limited range of substrates were examined, such labels can become misleading as more results are accumulated. For example, on the basis of **1a**, all of the yeast dehydrogenases investigated here would be classified as

L-selective; however, six produce primarily the D-alcohol from **1b** (Table I). Other examples of conflicting nomenclatures could also be cited. The bottom line is that the stereoselectivity observed depends on ***both*** the enzyme and the ketone substrate. It is not always easy to predict which substrate structural features will provoke a different active site-binding mode, and it was for this reason that we concentrated our efforts on devising rapid and simple methods to determine the outcomes of proposed reactions empirically. The original efforts focused on the *S. cerevisiae* genome since this was the first organism to be completely sequenced, and it was known to be a rich source of intracellular dehydrogenases. The studies summarized here were designed primarily as proof-of-principle, and their success invites further expansion, both within and outside the dehydrogenase arena. With the explosive growth in genome sequence data, the question is how to direct the search for new enzymes most efficiently.

One critical need is for reductases that mediate *si*-face hydride addition to small substrates such as ethyl acetoacetates (Fig. 4). In the absence of a polar group (e.g., as in **1b**), yeast dehydrogenases produce almost exclusively L-alcohols, the products of *re*-face hydride attack. As noted earlier, this predilection may be due to the enzymes' preference for glucose hydroxyl configurations in their natural carbohydrate substrates. If this is the case, dehydrogenases from organisms that assimilate less-common carbohydrates, whose substituent

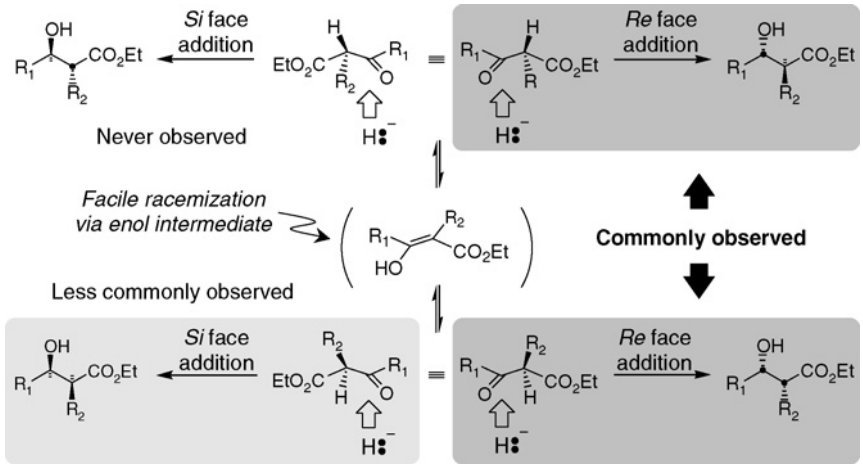


FIG. 4. Summary of stereoselectivities in β-keto ester reductions by bakers' yeast dehydrogenases.

configurations match D-*syn* and D-*anti* alcohols, may be useful. It may also be possible to reengineer the stereoselectivities of existing enzymes. Several strategies for making and recombining random mutations are available (Arnold, 1998; Crameri *et al.*, 1998). When coupled with a suitable high-throughput screen, these methods have proven very useful in altering enzyme stereoselectivities (May *et al.*, 2000; Park *et al.*, 2005; Reetz *et al.*, 2004). Although they have not yet been applied to dehydrogenases, there is no reason to believe that these methodologies would enjoy less success in this arena. Kazlauskas has recently pointed out the benefits of targeting mutations near the active site to achieve the most significant improvements (Morely and Kazlauskas, 2005). Recent results with yeast aldose reductases have hinted that stereoselectivity may be critically influenced by the Loop A region, making this protein segment an intriguing target (Kayser *et al.*, 2005).

Given the success of the genome library approach in providing reductases with a good substrate range and high stereoselectivities, the future of this genome-based approach seems bright. The sequence analysis can be carried out easily, which rapidly generates a list of candidate genes assembled. The actual gene cloning steps had been the most time-consuming; however, the recent commercialization of recombination-based cloning strategies allows an expression plasmid to be constructed from a polymerase chain reaction (PCR)-amplified gene in a single step. Our original studies utilized GST-affinity purification tags since the *S. cerevisiae* library had been constructed in this way. Other fusion partners (and even overexpression hosts) could also be employed, and these may be more appropriate for specific proteins. Commercial kits for coupled *in vitro* transcription/translation can now produce usable protein quantities directly from PCR-amplified DNA, and these could expedite the screening process to an even greater degree. With these improvements, the timeline for biocatalytic process development may become sufficiently short that enzymes will be used even more often in commercial applications.

ACKNOWLEDGMENTS

Work in the author's laboratory was made possible by generous financial support from the National Science Foundation (CHE-0130315). We also thank Dr Mark R. Martzen for supplying the original yeast GST-fusion protein clones. The real credit goes to the students and collaborators who participated in this work and brought the ideas to fruition.

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Process and Catalyst Design Objectives for Specific Redox Biocatalysis

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I. Introduction

Microorganisms are employed as biocatalysts in the food and dairy sector and also in pharmaceutical and chemical industries (Ghisalba, 2000; Liese *et al.*, 2000). An increasing number of pharmacologically active secondary metabolites are produced by microorganisms. Examples include antibiotics, steroids, or alkaloids for the treatment of infections, cancer, transplant rejection, and high cholesterol (Farnet and Zazopoulos, 2005; Mijts and Schmidt-Dannert, 2003). In the chemical industry, the recent trend toward environmental sustainability promotes the use of biomass (cellulose, starch, lignin, or sugars) as a renewable feedstock for the production of chemicals (Held *et al.*, 2000; Parales *et al.*, 2002; Werpy and Petersen, 2004). In a first step, fermentation products,

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ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 59 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(06)59003-3

such as ethanol, acetic acid, butanol, lactic acid, or propanediol, can be obtained (Bommarius and Riebel, 2004; Bothast and Schlicher, 2005; Ezeji *et al.*, 2004; Jeffries, 2005; Lee *et al.*, 2004; Liese *et al.*, 2000; Vink *et al.*, 2004). These compounds as well as oil components can serve as substrates for the synthesis of high-value-added biotransformation products, for example, (chiral) fine and specialty chemicals, synthons etc. (Bommarius and Riebel, 2004; Faber, 2004). Such biotransformations can be catalyzed by isolated enzymes as well as wild-type or recombinant microorganisms (over)producing the enzyme of interest (Duetz *et al.*, 2001; Ishige *et al.*, 2005; Matsuyama *et al.*, 2002; Sariaslani, 1989; Schmid *et al.*, 2001; Straathof *et al.*, 2002). The complex nature and the prevalent cofactor dependency of oxidoreductases constrict the use of isolated enzymes for redox biocatalysis (Bühler and Schmid, 2004; Faber, 2004). Thus, efforts toward the industrial application of oxidoreductases have mainly focused on whole-cell biocatalysis.

A crucial parameter in the development of a biocatalytic process is the productivity, which is defined by the yield of product per time and volume and influenced by the biocatalyst efficiency, by nonbiological factors, such as the quality of raw materials or substrates (upstream processing), and by the efficiency of product removal and purification (downstream processing) (Fig. 1A). One important factor determining biocatalyst efficiency is its specific activity described as the specific rate of product formation during biotransformation, for example, in units (μmol product per minute) per gram of cell dry weight (CDW). It depends on the synthesis, concentration, and activity of functional enzyme as well as on cofactor availability (Fig. 1A). Cell metabolism plays a key role by regenerating cofactors as donors or acceptors of reduction equivalents to support redox biocatalysis and for maintaining cell functionality, for example, resistance against toxic chemicals. Furthermore, not only the maximal performance but also the interaction and stability of cell metabolism, enzyme synthesis, and enzyme catalysis have to be considered for the evaluation of biocatalyst efficiency (Fig. 1B). Within this chapter, we discuss selected targets for intensification of redox bioprocesses (Fig. 2). To limit the scope, mass transfer of substrate/product across the cell envelope was omitted, although the biotransformation rate may be increased by alterations in the outer membrane (Ni and Chen, 2004, 2005; van Beilen *et al.*, 2003). Furthermore, aspects of enantioselectivity in whole-cell biotransformations are not considered, although cell physiology may influence the enantio-specificity of a transformation (Bertau, 2002).

Here, we discuss gene expression, cell metabolism, cofactor availability, oxygen transfer, and catalyst stability as possible targets for improving catalyst efficiency (Fig. 2). Integrating these aspects on a

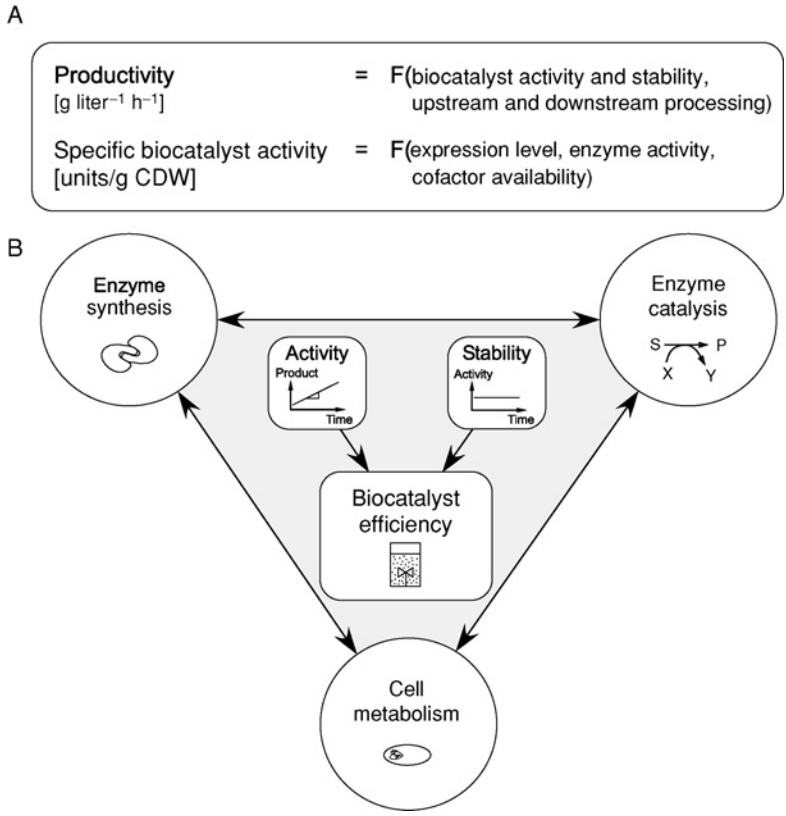

FIG. 1. Factors determining process and biocatalyst efficiency.

molecular, physiological, and reaction-engineering level allows increasing the productivity of bioprocesses. In this respect, the selection of organic solvents and recombinant host strains is critically discussed. In particular, the applicability of solvent-tolerant bacteria in two-liquid-phase biotransformations is evaluated.

II. Gene Expression

The efficiency of a microbial biocatalyst depends on the amount of functional enzyme that is produced and maintained during biotransformation (Fig. 1B). Wild-type bacteria often have to be cultivated on specific substrates in order to induce the synthesis of catabolic enzymes used for biotransformation. Such enzymes are usually encoded as single gene copies on the bacterial genome or on catabolic plasmids.

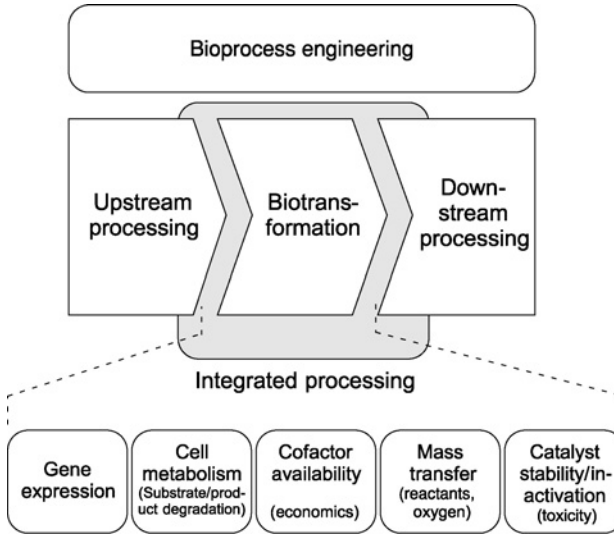

FIG. 2. Targets for bioprocess intensification.

This might result in the synthesis of relatively low intracellular enzyme concentrations. Today, recombinant DNA technologies allow multiplying the gene templates, either by introducing further gene copies into the genome or by providing them on expression vectors. In addition, the level of gene expression can be fine-tuned by introducing and engineering of regulatory sequences such as promoters, enhancers, or ribosome-binding sequences.

A. PROMOTERS

Various promoter sequences have been characterized and shown to be useful for the regulation of heterologous gene expression in *Escherichia coli* (Goldstein and Doi, 1995; Weickert *et al.*, 1996). A strong promoter provides a high expression level, which results in high enzyme concentrations. However, high-level expression is not always desired, since it represents a metabolic burden for the cells by draining the resources needed for growth and productive reactions. High concentrations of ''toxic'' proteins may even be harmful for the cells (Goldstein and Doi, 1995). Host bacteria can respond to the overproduction of membrane-bound enzymes by increased phospholipid synthesis, which may have negative effects on the physiology and morphology of the cell (Chen *et al.*, 1995a, 1996; Nieboer *et al.*, 1993,

1996). Unproductive recombinant enzyme activity, such as the uncoupling observed for many oxidoreductases, may lead to a loss of cofactors and in the case of oxygenases to an increased oxygen demand and the production of toxic hydrogen peroxide (Lee, 1999; Loida and Sligar, 1993). The use of inducible promoters with tunable expression levels is, therefore, often preferred to constitutive gene expression for biotransformations with metabolically active cells.

The best studied promoter is that controlling the expression of the *lac* operon in *E. coli*. The *lac* system was among the first regulatory systems studied and is still frequently used as a tool in biotechnology, for example, in pUC-derived vectors (Gronenborn, 1976; Vieira and Messing, 1982). However, the expression level reached via the *lac* promoter is relatively low. Derivatives of the *lac* promoter, such as *tac*, *trc*, or T7 promoters, provide higher expression levels but, as the *lac* promoter, show a relatively high basal expression in the absence of inducer (''leakiness'') (Jonasson *et al.*, 2002; Weickert *et al.*, 1996) (Table I). Heterologous gene expression under the control of *ara* (or *bad*), *rha*, or *alk* promoter systems is ''tighter'' and can be finer tuned by titration of inducing agents (Bühler *et al.*, 2000; Guzman *et al.*, 1995; Haldimann *et al.*, 1998; Panke *et al.*, 1999b; Perez-Perez and Gutierrez, 1995). This was exploited in a number of biotransformations (Bühler *et al.*, 2003b; Doig *et al.*, 2002; Panke *et al.*, 2002) and for the production of heterologous enzymes on a large scale (Doig *et al.*, 2001; Hofstetter *et al.*, 2004). Based on *alk* and *ara* promoters, vectors have been constructed that provide various selection markers and polylinkers for an optimized use in gene expression (Cronan, 2005; Smits *et al.*, 2001).

Another advantage of gene expression under the control of *alk*, *ara*, or *rha* promoter systems is the induction by relatively cheap chemicals, such as octane or dicyclopropyl ketone, arabinose, or rhamnose, respectively. In contrast, *lac*, *tac*, *trc*, and T7 promoters are induced by isopropyl-β-D-thiogalactoside (IPTG), which is not ideal due to rather high costs for IPTG. Lactose, the natural inducer of the *lac* operon, can be used as cheap alternative for IPTG (Neubauer and Hofmann, 1994). However, its concentration and thus the expression level can change during biotransformation since it is metabolized by the cells. Yet, IPTG purchase prizes for quantities used in large-scale biotransformations are an order of magnitude lower than indicated in catalogues of chemical purchasers (Dr. M. Schedel, Bayer HealthCare AG, Wuppertal, Germany, personal communication). In addition, already 50–100 μM IPTG can be sufficient for full induction (Baneyx, 1999). Thus, induction by IPTG is not necessarily a significant cost factor for the synthesis of high-value-added products on multicubic meter scales.

TABLE I

PROPERTIES OF PROMOTERS COMMONLY USED IN BIOCATALYSIS (EXTENDED FROM WEICKERT *ET AL.*, 1996)

Promoter	Inducer[a]	Advantages	Disadvantages
lac	IPTG	Many vectors available (e.g., pUC derivatives)	Relatively low expression level; ''leaky'' expression; inducer rather expensive; repressed by glucose
tac or *trc*	IPTG	Many vectors available; high-level expression; induction level can be titrated	''Leaky'' expression; inducer rather expensive
T7	IPTG	Many vectors available (e.g., pET derivatives); high-level expression	''Leaky'' expression; difficult to achieve high cell densities; inducer rather expensive
alk	DCPK, octane, etc.	High-level expression; induction level can be titrated; ''tight'' regulation; cheap inducer	Few vectors available
ara or *rha*	Arabinose or rhamnose	Wide range of induction levels possible; ''tight'' regulation; rapid induction/repression; cheap inducer	Few vectors available; catabolite repression by glucose

IPTG: isopropyl-β-D-thiogalactoside; DCPK: dicyclopropyl ketone.
[a]Only induction by chemicals is considered.

Some promoter systems induce gene expression as a function of temperature, pH, or as a result of the depletion of a certain nutrient in the growth medium (Chou *et al.*, 1995; Goldstein and Doi, 1995; Weickert *et al.*, 1996). For biocatalysis, however, induction by the addition of a chemical is simpler than by its removal or by temperature or pH shifts. The optimization of reaction conditions, such as pH and temperature, generally aims at maximizing catalytic performance rather than gene expression.

Ongoing research with respect to promoter engineering and vector design will result in the development of new and improved expression systems with higher expression levels, reduced leakiness, and better regulation (Alper *et al.*, 2005; Mertens *et al.*, 1995a,b; Royo *et al.*, 2005; Weickert *et al.*, 1996). New expression vectors will be useful for more efficient syntheses of functional enzymes, and thus may lead to higher

yields of value-added products in processes based on recombinant whole-cell biocatalysts.

B. Limitations of Protein Overproduction

Gene overexpression in a heterologous host does not always result in higher specific activities as compared to the native host. In other words, specific enzyme activities in recombinants are often lower than in the wild type, which can be explained by differences in the molecular environment in different strains. Critical factors include genetic stability, transcriptional or translational regulation, RNA or protein stability, as well as protein folding, processing, and assembly (Staijen *et al.*, 1997, 2000; Zeyer *et al.*, 1985).

''Self-cloning,'' the overexpression of the gene of interest in the native host, might be a suitable strategy to overcome problems associated with the strain specific expression machinery (Duetz *et al.*, 2001). Broad host range vectors can be used for gene expression in bacteria different from *E. coli* (Arai *et al.*, 1991; Graupner and Wackernagel, 2000; Mermod *et al.*, 1986; Rangwala *et al.*, 1991; Toukdarian, 2004; Yen, 1991). Since most of these vectors are present at low copy numbers, shuttle vectors with an increased plasmid copy number were constructed (Smits *et al.*, 2001; Tao *et al.*, 2005). These plasmids are useful for gene expression in various hosts, but their suitability for biocatalysis under large-scale process conditions, including aspects such as genetic and segregational stability, is hardly investigated so far.

Stable biocatalysts have been constructed by placing genes from *Pseudomonas* strains via transposons into the chromosome of strains belonging to the same genus or species (Hüsken *et al.*, 2001a; Panke *et al.*, 1999a; Wery *et al.*, 2000; Wierckx *et al.*, 2005). Although only one set of styrene monooxygenase genes (*styAB*) from *Pseudomonas* sp. strain VLB120 was introduced into the genome of *P. putida* KT2440, the specific styrene oxidation rate of engineered *P. putida* SAB was higher than the rate obtained with recombinant *E. coli* JM101(pSPZ10) overexpressing the genes on a multicopy number plasmid (Panke *et al.*, 1999a). In contrast, styrene monooxygenase activity was not detectable in *E. coli* strains engineered in the same way, and harboring one set of *styAB* genes on the chromosome.

In another study, the potential drawback of low enzyme concentrations due to a single chromosomal gene insertion was addressed by placing up to 150 copies of mercury resistance and toluene dioxygenase genes in the genome of *Deinococcus radiodurans*, the most

radiation-resistant organism known (Brim *et al.*, 2000). The resulting engineered bacterium can be employed for bioremediation of radioactive wastes containing ionic mercury and toluene.

Metagenome screening suggests that only a minor part of identified genes can be expressed in *E. coli* hosts (de Lorenzo, 2005; Gabor *et al.*, 2004). Thus, recombinants based on host strains providing appropriate molecular environments for gene expression will be essential to exploit newly identified enzyme activities for biocatalysis. Yet, further developments of molecular biological tools and an in-depth physiological characterization are required to make alternative host strains accessible for recombinant biocatalysis. Here, the PlugBug concept of DSM (Heerlen, The Netherlands) represents a promising approach, which makes use of only a limited number of different, well-characterized host strains for the synthesis of the majority of biocatalytically produced DSM products.

III. Cell Metabolism

Residual substrates or side-products frequently complicate downstream processing because of their similar physiochemical properties as compared to the product. Substrates and products can be either chemically, thermally, or biologically unstable under reaction conditions leading to side-product formation and product degradation. Biological instability may be caused by the metabolic machinery of the microbial host leading to uncontrolled substrate or product conversion by host enzymes.

Normally, bacteria are cultivated in aqueous, often aerobic environments with temperature and pH kept within physiological ranges. Biocatalytic production of compounds that are chemically unstable under these conditions, such as epoxides (Hofstetter *et al.*, 2004; Panke *et al.*, 2000) or catechols (Held *et al.*, 1999; Meyer *et al.*, 2003; Wery *et al.*, 2000), requires suitable reaction-engineering solutions such as the *in situ* extraction of the reactants from the cell broth with a protective organic phase (Lye and Woodley, 1999). This organic phase may either be a liquid or a solid, which stabilizes substrate and product as well as protects cells from toxic effects (Section VI.A).

On the one hand, catabolic pathways of bacteria able to metabolize a broad range of aromatic chemicals are interesting for biocatalytic applications (Jimenez *et al.*, 2002; Wackett, 2003). An example is the use of *P. putida* for the cometabolic production of a variety of heteroaromatic carboxylic acids on cubic meter scale by Lonza AG (Kiener, 1992; Schmid *et al.*, 2001; Visp, Switzerland). On the other hand,

the catabolic potential of such microbial strains can result in side-product formation or breakdown of substrate/product during biocatalysis.

The inactivation of disturbing enzyme activities or the cloning of genes encoding the enzymes of interest in microbial hosts, which provide a ''neutral'' enzymatic background are strategies to avoid biological product instability.

A. Avoidance of Side-Product Formation

Additives or inhibitors may be used to reduce enzymatic side-product formation, but in bioprocesses, they are seldom applied. Reasons may be higher costs or the associated increase in complexity of such a process (Bühler and Schmid, 2004). Nevertheless, this approach is well documented for the production of phenylacetylcarbinol from pyruvate and benzaldehyde by pyruvate decarboxylase during yeast fermentation (Oliver *et al.*, 1999), which is one of the earliest biotechnological processes relevant for the chemical industry (Liese *et al.*, 2000). Significant amounts of benzaldehyde are lost as substrate for pyruvate decarboxylase due to the activity of alcohol dehydrogenases, which reduce benzaldehyde to benzyl alcohol (Nikolova and Ward, 1991). The most effective method to prevent the formation of benzyl alcohol from benzaldehyde during phenylacetylcarbinol production is the addition of alternative proton acceptors beside benzaldehyde, such as colloidal sulfur or acetaldehyde. However, due to the high toxicity of sulfur, only acetaldehyde was applied in fermentative phenylacetylcarbinol production. Conversion of benzaldehyde to phenylacetylcarbinol increased from 40% to 70% accompanied by a proportional decrease of benzyl alcohol formation (Oliver *et al.*, 1999; Smith and Hendlin, 1954). The use of nicotinic acid analogues to compete with NADH for enzyme active sites or iodoacetic acid as an inhibitor of sulfhydryl-containing enzymes (such as alcohol dehydrogenases) brought only little success since these compounds inhibited metabolically important enzymes. It is noteworthy that cell-free bioprocesses yield higher phenylacetylcarbinol productivities since no reduction of benzaldehyde to benzyl alcohol occurs when cell-free extracts or purified pyruvate decarboxylase are used as biocatalysts (Rosche *et al.*, 2002).

The use of mutants without interfering side-activities is another possibility to reduce enzymatic substrate/product degradation during biotransformation (Knop *et al.*, 2001). This method enables the design of biocatalysts, which accumulate an intermediate product as

a result of an interrupted metabolic pathway (Bosetti *et al.*, 1992). As an example, an *E. coli* mutant was generated, which is completely blocked in its ability to metabolize pyruvate as a result of the deletion of genes coding for pyruvate dehydrogenase, pyruvate formate lyase, pyruvate oxidase, PEP synthetase, and lactate dehydrogenase (Zelic *et al.*, 2003). Glucose was converted to pyruvate, which accumulated to concentrations higher than 62 g liter^{-1} in fed-batch cultivations with acetic acid as sole carbon source. However, genetic engineering can be complicated if substrate and/or product degradation is catalyzed by multiple enzyme activities and enzymes with relaxed substrate specificities, for example, dehydrogenases. Comparative analysis of the *P. putida* KT2440 genome revealed up to 40 putative dehydrogenases, of which the substrate specificity is unknown (Nelson *et al.*, 2002). Thus, the generation of mutants, which are not able to degrade a certain substrate/product, might require multiple deletions of unspecific enzyme activities. This raises the question whether such genetically engineered biocatalysts still provide the metabolic fitness to efficiently catalyze energy-dependent redox reactions and to cope with harsh process conditions.

B. Use of Recombinant Host Strains

Bacteria expressing heterologous genes are applied for the biocatalytic production of a broad range of value-added chemicals. Next to bacteria, yeast species are also used as recombinant biocatalysts and are of particular interest for glycosylations (Wohlgemuth, 2005). Recombinant whole-cell biocatalysis has developed to a standard technology, which frequently uses *E. coli* strains as bacterial hosts (Schmid *et al.*, 2001). The high popularity of this host bacterium may be due to its excellent accessibility by molecular biology methods, high achievable growth rates and biomass yields on minimal medium with cheap carbon sources, nonpathogenicity, and its high cofactor regeneration potential. In addition, the PCR technology allows accessing the genetic information of currently more than 250 bacterial genomes (http://www.tigr.org/), which can be expressed in *E. coli* by numerous compatible gene expression systems (Baneyx, 1999; Yasueda and Matsui, 1994).

E. coli is mainly known as an inhabitant of the intestines of warm-blooded animals, where it is not expected to get in touch with a broad range of, for example, aromatic compounds. Thus, *E. coli* strains are often assumed to have only a low tendency to degrade aromatic substrates and products and to provide a ''neutral'' enzymatic background

activity in recombinant biocatalysis. However, Diaz *et al.* (2001) summarized *E. coli* catalyzed reactions involved in the degradation of many aromatic compounds, which illustrates that this bacterium is not a catabolically ''empty box.'' An example is *E. coli* JM101, which showed an even faster accumulation of aromatic amines, side-products of the biocatalytic oxygenation of *m*-nitrotoluene, than two *P. putida* strains (Meyer *et al.*, 2005). For the selective oxidation of nitro aromatics by xylene monooxygenase in recombinant *E. coli* JM101, side-product formation not only reduced production yields but the accumulated aromatic amines also inhibited xylene monooxygenase activity. This example shows that, in contrast to the general opinion, the enzymatic background activity of *E. coli* is capable of degrading a number of aromatic compounds and, therefore, is not a universally applicable host strain for biotransformations. (Multiple) knockout mutations to reduce or delete interfering enzyme activities, however, can threaten metabolic activity of the cells, which is a prerequisite for maintaining general metabolic function, cofactor regeneration, and continuous synthesis of recombinant enzymes. The systematic screening and classification of microorganisms with an enzymatic background activity that is suitable for catalysis of a desired reaction type thus represents a promising strategy to overcome substrate and product degradation.

IV. Cofactor Availability

Most oxidoreductases are cofactor dependent and reactions catalyzed by such enzymes are usually associated with the consumption of stoichiometric amounts of NAD(P/H) (Harayama *et al.*, 1992). In order to make biotransformations with isolated enzymes or preparations from crude extracts economically feasible, enzymatic or non-enzymatic cofactor regeneration systems are required (Faber, 2004; Hollmann and Schmid, 2004; Wichmann and Vasic-Racki, 2005). Whole-cell biotransformations generally do not require additional regeneration systems, since cofactors are regenerated by the metabolic activity of the microbial catalyst. To make such whole-cell processes interesting as alternatives to classical organic syntheses, high productivities are required (Schmid *et al.*, 2001). This raises the question about a possible limitation of whole-cell redox biocatalysis by the cofactor regeneration capacity of cell metabolism.

Seven different bacterial species cultivated aerobically in batch mode at 30°C were recently investigated for pathways involved in glucose metabolism (Fuhrer *et al.*, 2005). In order to evaluate the

potential of these strains to serve as hosts for NAD(P)H-dependent redox biocatalysis, we used the reported growth physiological data to estimate their reductive cofactor regeneration capacity (Table II). For the calculation, the elemental ratio of biomass was assumed to be $CH_{1.67}\ O_{0.27}N_{0.2}$ (Duetz *et al.*, 2001). In order to simplify the estimation, every molecule of glucose not metabolized for biomass or acetic acid production was assumed to lead to the generation of eight molecules of NADH and two molecules of NADPH. Every molecule of accumulated acetic acid was assumed to result in the generation of two molecules of NADH. However, cofactor specificity of enzymes involved in NAD(P)H generation may vary among different species. Since transhydrogenases can convert NADPH to NADH and vice versa by transferring electrons directly from NADPH to NAD^+ or from NADH to $NADP^+$ (Jackson, 2003; Sauer *et al.*, 2004), the total pool of oxidized nicotinamid cofactors may be available, for example, for NADH-dependent biocatalysis. Resulting (total) NAD(P)H regeneration rates during growth of the different bacterial species were between 126 and 2218 U/g CDW (Table II). This is in accordance with earlier calculations, which proposed a rate of 720 U/g CDW for NADH regeneration (Duetz *et al.*, 2001). The NAD(P)H regeneration rate of *E. coli* was estimated to be about 575 U/g CDW, which is in the same order of magnitude as the maximum specific activities of oxygenases that have been reached in practice (about 500 U/g CDW; Duetz *et al.*, 2001). Under the assumption that nongrowing cells have the same capacity for glucose catabolism, the glucose that is metabolized for biomass formation would become available for cofactor regeneration (Table II). As a result, the estimated NAD(P)H regeneration rate for nongrowing *E. coli* cells increases to 1125 U/g CDW. *Paracoccus versutus*, a facultative autotroph belonging to the α-proteobacteria, showed the highest specific glucose uptake rate during growth, which resulted in about three- to fourfold higher estimated NAD(P)H regeneration rates than for *E. coli* (Table II). Although this species is not yet exploited for biocatalytic applications, its enormous TCA cycle activity makes it interesting as an alternative host for future applications with high demands for cofactor regeneration. In spite of *S. meliloti*, *A. tumefaciens*, and *R. sphaeroides* showed a lower NAD(P)H regeneration potential, other α-proteobacteria might be found with regeneration potentials comparable to *Paracoccus versutus* and might promote the use of strains, such as *Sphingomonas* sp., as biocatalysts for oxidative biotransformations (Li *et al.*, 2001).

The simplified calculation of NAD(P)H regeneration rates considers neither maintenance energy demands nor energy consumption as a result

TABLE II

GROWTH PHYSIOLOGY AND NAD(P)H REGENERATION RATES OF DIFFERENT BACTERIA CULTIVATED AEROBICALLY ON GLUCOSE IN BATCH-MODE

					Estimated cofactor regeneration rates[c]							
					During growth				Without growth[c]			
					NADH		NADPH		NADH		NADPH	
Organism[a]	Max. spec. growth rate[b] [hour^{-1}]	Biomass yield[b] [g CDW/ g gluc.]	Glucose uptake rate[b] [mmol g^{-1}hour^{-1}]	Acetic acid accumulation rate[b] [mmol g^{-1}hour^{-1}]	[mmol g^{-1}hour^{-1}]	[U/g][d]	[mmol g^{-1}hour^{-1}]	[U/g][d]	[mmol g^{-1} hour^{-1}]	[U/g][d]	[mmol g^{-1} hour^{-1}]	[U/g][d]
P. fluorescens	0.49	0.44	4.5		13.7	228	3.4	57	36.0	600	9.0	150
S. meliloti	0.17	0.41	2.3		7.8	129	1.9	32	18.4	307	4.6	77
A. tumefaciens	0.30	0.41	4.1		13.8	231	3.5	58	32.8	547	8.2	137
Paracoccus versutus	0.70	0.21	18.9		106.4	1774	26.7	444	151.2	2520	37.8	630
R. sphaeroides	0.15	0.41	1.8		6.1	101	1.5	25	14.4	240	3.6	60
E. coli	0.39	0.30	7.8	3.5	29.0	483	5.5	92	55.4	923	12.1	202
B. subtilis	0.30	0.35	4.8	2.1	15.3	254	2.8	46	34.2	570	7.5	125

[a]Organisms: *Pseudomonas fluorescens* 52-1C, *Sinorhizobium meliloti* (DSMZ 1981), *Agrobacterium tumefaciens* C58, *Paracoccus versutus* A2, *Rhodobacter sphaeroides* ATH 2.4.1, *Escherichia coli* MG1655, *Bacillus subtilis* 168 trpC2.

[b]Data obtained from Fuhrer *et al.* (2005).

[c]See text for details.

[d]One unit is defined as one μmol NAD(P)H regenerated per minute.

of stress responses, which may appear under bioprocess conditions. High concentrations of substrates, products, or organic solvents in the cell broth can have toxic effects with membrane disintegration being the most pronounced mechanism of solvent toxicity (Sikkema *et al.*, 1995) (Section VI). Thus, uncoupling of the proton motive force eventually reduces the amount of cofactors available for biocatalysis. A recent study on stereospecific styrene epoxidation catalyzed by recombinant *E. coli* containing styrene monooxygenase from *Pseudomonas* sp. strain VLB120 suggests that a product toxicity induced cofactor limitation affected the styrene oxide productivity under process conditions (Park, 2004).

Uncoupling of energy demands for biotransformation from demands for cell growth is an interesting issue in biocatalysis. Unfortunately, many processes involving oxygenases are only productive when growing cells are used as biocatalysts (Bühler *et al.*, 2003a,b; Favre-Bulle and Witholt, 1992; Favre-Bulle *et al.*, 1991, 1993). Possible reasons include decreasing intracellular oxygenase concentrations due to changes in the regulation of gene expression (Ishihama, 1997), protein stability (Gottesman, 2003), or cell metabolism and physiology (membrane stability). A decreasing metabolic activity may affect NAD(P)H regeneration when cells enter the stationary growth phase. Here, metabolic engineering may contribute to a better understanding and enable the manipulation of metabolic fluxes in order to channel energy to biocatalysis instead of biomass synthesis (Lee *et al.*, 2003; Poulsen *et al.*, 2005; Wandrey, 2004).

Another factor which reduces cofactor availability is the interference of enzymatic background activity with biocatalysis. Such an interference was observed, when recombinant *E. coli* containing xylene monooxygenase were used for the successive oxidation of toluenes and xylenes to the corresponding alcohols, aldehydes, and acids (Bühler *et al.*, 2000; Maruyama *et al.*, 2003; Meyer *et al.*, 2005). In these applications, the oxidation of alcohols to the corresponding aldehydes catalyzed by the monooxygenase was counteracted by the reduction of aldehydes to alcohols catalyzed by dehydrogenases from the *E. coli* host. This ''futile cycle'' not only reduced the aldehyde formation rate but also acted as a sink for reduced cofactors, since both reactions use stoichiometric amounts of NADH (Bühler *et al.*, 2000; Harayama *et al.*, 1992; Meyer *et al.*, 2005). On the other hand, the involvement of both oxygenases and dehydrogenases for the catalysis of successive oxidations uncouples biocatalysis from cofactor regeneration by the host metabolism. The NADH consumption due to the oxygenase-catalyzed reaction, thereby, is compensated by NADH generation caused by the dehydrogenase-catalyzed oxidation. This approach was used in an environmental

application for the biodegradation of 2-chlorotoluene by engineered pseudomonads (Haro and de Lorenzo, 2001) and in synthetic applications for the production of 3-methylcatechol from toluene by genetically engineered *P. putida* strains (Hüsken *et al.*, 2001a; Wery *et al.*, 2000).

Overall, specific activities do not seem to be limited by cofactor availability in current industrial processes. However, this issue will certainly be of importance in future applications using optimized biocatalysts with higher specific activities or in applications running under conditions, which impose an additional burden on the metabolism of the microorganisms involved.

V. Oxygen Transfer

High oxygen transfer rates are important for all aerobic bioprocesses and crucial for biocatalytic reactions with molecular oxygen as a substrate. Oxygenases, for instance, incorporate molecular oxygen into a second substrate. In whole-cell biotransformations, oxygen has to be transferred from air to the enzyme via the cultivation medium and the cell envelope. The oxygen solubility is relatively low in water, which can lead to oxygen limitation in oxygenase-based biotransformations involving high concentrations of (respiring) cells, because generally respective K_m values of oxygenases are significantly higher than those of the cytochrome complexes in the electron-transfer chain (Duetz *et al.*, 2001). Technically, oxygen transfer can be improved by increasing the stirring rate (which results in the formation of smaller air bubbles), the oxygen content in the gas influx, or the pressure inside the reactor (Onken and Liefke, 1989; Schmid *et al.*, 1999), or by the addition of a second liquid phase with a higher oxygen solubility than water (oxygen vectors) (Giridhar and Srivastava, 2000; Rols and Goma, 1989; Rols *et al.*, 1990). Nevertheless, especially in large-scale applications, oxygen transfer can become limiting for cell growth and/or biocatalysis. In a stirred-tank reactor on a laboratory scale aerated with air, maximum oxygen transfer rates in the range of 300 to 400 mM $hour^{-1}$ have been reached. Industrial, 100-cubic meter stirred-tank reactors show significantly lower oxygen transfer rates, which are typically below 100 mM $hour^{-1}$ (Held *et al.*, 2000; Schmid, 1997).

Uncoupling of oxygen demands used for respiration during growth from demands for oxygenase catalysis might be achieved by biotransformation in the stationary phase or in recombinant hosts with low endogenous respiration activities, which also may influence NAD(P)H availability. Here, the question is to what extent NAD(P)H regeneration is driven by demand, especially in resting cells (Section IV).

An interesting approach for enhanced oxygen supply is the addition of hydrogen peroxide to microbial cultures in the presence of catalase, which converts hydrogen peroxide to water and molecular oxygen (Nies and Schlegel, 1984; Schlegel, 1977). Catalase is synthesized by many microorganisms or it can be added separately to the culture. Using this approach, cells incubated in an anaerobic nitrogen atmosphere grew at the identical rates as cells incubated under standard aerobic conditions. Intracellular specific catalase activities for the cleavage of hydrogen peroxide are relatively high (e.g., *P. putida*, 50,000 U/g CDW; Schlegel, 1977). The fast turnover of toxic hydrogen peroxide might therefore explain the absence of growth inhibition. The addition of hydrogen peroxide can be regulated as a function of dissolved oxygen tension in the culture broth or of oxygen partial pressure in the off-gas of the bioreactor (Sonnleitner and Hahnemann, 1997). This setup was useful for the production of gluconic acid from glucose by *Aspergillus niger* (Rosenberg *et al.*, 1992) and might be especially suitable for biotransformations requiring low aeration rates to minimize the loss of volatile reactants, foam formation, and explosion hazard (Sonnleitner and Hahnemann, 1997). Oxygen transfer to cells susceptible to hydrodynamic stresses can be performed by bubble-free aeration across membranes (Lütz *et al.*, 2005). Application of hydrogen peroxide and catalase thereby may represent an interesting alternative, although the hydrogen peroxide sensitivity of mammalian cells may preclude such a strategy.

In contrast to oxygenase-catalyzed reactions, hydroxylations catalyzed by peroxidases and dehydrogenases do not require molecular oxygen as a cosubstrate, thus turning them into particularly interesting candidates for the catalysis of hydroxylations. Peroxidases catalyze a large variety of hydroxylations and thereby produce one molecule of water from hydrogen peroxide (or alcohol in the case of organic peroxide driven peroxidations) as a coproduct (Adam *et al.*, 1999; van Deurzen *et al.*, 1997b; van Rantwijk and Sheldon, 2000). A further advantage of peroxidases is that they need no regeneration of cofactors such as NAD(P)H. However, a major shortcoming is the low operational stability of peroxidases, generally resulting from peroxide induced deactivation (van de Velde *et al.*, 2001). An example is the facile oxidative deterioration of the porphyrin ring in heme-dependent peroxidases such as the frequently used chloroperoxidase (van Deurzen *et al.*, 1997a; van de Velde *et al.*, 2000).

Dehydrogenases can incorporate oxygen derived from water. As an example, the bacterial hydroxylation of 6-methylnicotinic acid to 2-hydroxy-6-methylnicotinic acid was performed under aerobic but

oxygen-limited conditions (Tinschert *et al.*, 1997). Hydroxylations of a variety of *N*-heteroaromatic carboxylic acids have been described (Fetzner, 1998, 2000; Hille, 1996; Kaiser *et al.*, 1996; Tinschert *et al.*, 1997; Wieser *et al.*, 1997) and were exploited for synthetic applications in industry (Kulla, 1991; Schmid *et al.*, 2001). However, it is noteworthy that molecular oxygen is one proposed electron acceptor in such dehydrogenase-catalyzed reactions, which might either impair or prohibit the uncoupling of biooxidations from oxygen transfer rates (Stephan *et al.*, 1996).

VI. Catalyst Stability and Inactivation

The productivity of a process is significantly influenced by the stability and robustness of the microbial biocatalyst under reaction conditions (Fig. 1B). The toxicity of substrate, product, or organic solvents involved in biocatalysis thereby plays a major role. The integration of organic chemicals (also referred to as organic solvents) in the lipid bilayer impairs structure and function of the membrane and ultimately leads to cell death (Sikkema *et al.*, 1994, 1995). The tendency of organic solvents to partition into membranes was found to correlate with their partitioning into the octanol phase of a defined octanol–water mixture (Seeman, 1972; Sikkema *et al.*, 1995). The logarithm of the octanol–water partition coefficient ($\log P_{ow}$) is therefore a useful measure for the assessment of cell-toxicity of organic solvents (Laane *et al.*, 1987). It was found that the toxicity tends to increase with a decreasing $\log P_{ow}$. A solvent with a $\log P_{ow}$ below 4 is generally considered to prevent growth of non-solvent-tolerant strains. Although organic solvents with a $\log P_{ow}$ over 4 partition better into membranes than low $\log P_{ow}$ solvents, their aqueous solubility is not high enough to cause lethal membrane concentrations (de Bont, 1998). Some chemicals provoke additional toxic effects, which are independent from the tendency to accumulate in membranes. As an example, nitro or amine groups render aromatic chemicals more toxic for microorganisms than predicted based on their hydrophobicity (Donlon *et al.*, 1995). Next to this molecular toxicity, cells can be affected by the presence of a second liquid phase of an organic solvent (phase toxicity) via a reduced substrate mass-transfer from the aqueous phase to the cell or by extraction of essential nutrients from the cell broth or membrane components from the cell envelope into the organic phase (Bar, 1986; Hocknull and Lilly, 1988).

A wide range of cytotoxic chemicals, such as phenol, catechol, toluene, styrene, xylenes, and derivatives thereof, are highly interesting

starting compounds in redox biocatalysis. Reaction engineering on the one hand and the use of more robust biocatalysts on the other hand are approaches, which make these substrates accessible for whole-cell biocatalysis.

A. Two-Liquid-Phase Biotransformation

In order to handle the toxicity of low-logP_{ow} reactants during biotransformation, a second, water-immiscible extractive phase can be introduced, which consists either of a solid or a liquid. Solid phases, for example, hydrophobic resins or capsules with a hydrophobic core, can be used for continuous product extraction, thereby effectively maintaining product concentrations in the cell broth at subtoxic levels (Hecht *et al.*, 1987; Held *et al.*, 1999; Lye and Woodley, 1999; Meyer *et al.*, 2003; Nielsen *et al.*, 1988; Stark *et al.*, 2003; Vicenzi *et al.*, 1997; Voser, 1982; Wang *et al.*, 1981). Ideally, second liquid phases serve as a sink for products and as a substrate reservoir. Thus, high concentrations of cytotoxic substrates and products can be present in emulsions consisting of a high-logP_{ow} organic solvent and an aqueous medium. This method not only minimizes toxic effects for the cells in the aqueous phase, but can also reduce substrate/product inhibition of biocatalytically relevant enzymes, thus favorably shifting the reaction equilibrium toward product formation or increasing oxygen transfer to the cells (Brink and Tramper, 1985; Leon *et al.*, 1998; Rols and Goma, 1989). Two-liquid-phase biotransformation setups with dioctylphthalate (logP_{ow}, 9.6) or medium- to long-chain alkanes, such as hexadecane (logP_{ow}, 8.8) or octane (logP_{ow}, 4.5), as second phases were exploited for selective biooxidations resulting in a wide number of products, including aromatic or aliphatic epoxides, alcohols, aldehydes, and acids as well as dihydrodiols and catechols (Bühler and Schmid, 2004). The production of 1-octanol from octane in an integrated two-liquid-phase bioprocess was evaluated with respect to reactor design and product recovery (Mathys *et al.*, 1999).

The extraction potential of very hydrophobic organic solvents is reduced for a number of low-logP_{ow} chemicals. In a hexadecane–water mixture, chemicals, such as phenol, cresol, or benzyl alcohol, partition preferably to water, whereas in octanol–water they partition preferably to octanol (Table III adapted from Abraham *et al.*, 1994). Thus, for the production of these chemicals, the use of organic solvents with a relatively low logP_{ow}, such as octanol, could be more efficient as compared to hexadecane or dioctylphthalate. Such low-logP_{ow} solvents, however, are toxic to ''normal'' bacteria. A two-liquid-phase biotransformation

TABLE III

PARTITION COEFFICIENTS OF CHEMICALS IN TWO-LIQUID-PHASE SYSTEMS CONSISTING OF WATER AND DIFFERENT ORGANIC SOLVENTS (ADAPTED FROM ABRAHAM *ET AL.*, 1994)

	Logarithm of partition coefficient (logP)[a]		
Solute	Octanol–H_2O ($logP_{ow}$, 3.1)	Cyclohexane–H_2O ($logP_{ow}$, 3.4)	Hexadecane–H_2O ($logP_{ow}$, 8.8[b])
Pentane	3.3	4.1	3.8
o-Xylene	3.1	n. a.	3.3
Styrene	3.0	n. a.	3.0
Toluene	2.7	3.0	2.7
p-Cresol	2.0	−0.24	−0.23
Benzoic acid	1.8	−0.78	n. a.
Phenol	1.5	−0.85	−1.0
Benzaldehyde	1.5	1.2	1.0
Benzyl alcohol	1.2	−0.54	−0.53
Catechol	0.9	−2.7	n. a.

[a]LogP values are means of observed and calculated partition coefficients given by Abraham *et al.* (1994).
[b]Data from Laane *et al.* (1987).
n. a.: not available.

system based on octanol as a second phase thus requires bacteria that can withstand these extreme conditions (Section VI. B).

How can the solvent with the best extraction properties for a certain chemical be identified? Partition coefficients of a variety of chemicals in different organic solvent–water mixtures represent one useful source to estimate the suitability of a solvent as a second phase for the extraction of (groups of) chemicals (Abraham *et al.*, 1994). The $logP_{ow}$ values of octanol and cyclohexane are 3.1 and 3.4, respectively, and thus quite similar. However, the extraction properties of cyclohexane for compounds, such as phenol or benzyl alcohol, are more comparable to the properties of hexadecane ($logP_{ow}$, 8.8) than to those of octanol, which may be explained by the nonpolar character of cyclohexane (Table III). This shows that partitioning behavior is not only determined by the $logP_{ow}$ but also is influenced by hydrogen bond formation, molecule size, and polarity of both solvent and solute (Abraham *et al.*, 1994). Solutes with carbonyl substituents have often similar partition coefficients in mixtures with octanol, cyclohexane, and hexadecane, whereas the corresponding partition coefficients of

solutes with hydroxyl- and carboxyl-groups are different. Also the position of substitutions can have a significant influence. As an example, the nitrophenol isomers have similar partition coefficients in an octanol–water mixture, whereas in a hexadecane–water mixture, the aqueous 2-nitrophenol concentration is about 700- and 2300-fold lower than the respective aqueous 3-nitrophenol and 4-nitrophenol concentrations (Abraham *et al.*, 1994). In addition, styrene and styrene oxide are more efficiently extracted by dioctylphthalate as second liquid phase than by hexadecane (Panke *et al.*, 2000), which can be explained by the chemical similarity (aromaticity) of the solutes and dioctylphthalate.

It is evident, that partition coefficients of chemicals determined in an organic solvent–water mixture do not necessarily represent their partitioning behavior observed in an organic solvent–medium mixture (Hüsken *et al.*, 2001b). Differences between theoretical estimations and reality may be even more pronounced for partitioning in a solvent–medium–cells mixture.

With regard to practical applications, further aspects beside extraction properties of an organic solvent have to be taken into account. Organic solvents can provoke many undefined physical phenomena with undesired side effects such as biomass clotting, cell aggregation at the liquid–liquid interface, loss of catalytic activity (via, e.g., enzyme inhibition), medium component accumulation in the organic phase, and slow coalescence (Bertau and Scheller, 2003; Brink and Tramper, 1985; van Sonsbeek *et al.*, 1993). These effects might be enforced or diminished depending on the strain used as a biocatalyst, for example, bacteria producing surface active compounds may provoke the formation of stable emulsions (Cooper and Zajic, 1980; de Smet *et al.*, 1983). Biomass clotting and aggregation or solvent emulsification and dispersion can complicate the determination of biomass concentration by spectrophotometric means during a two-liquid-phase biotransformation (Aono *et al.*, 2001; Hüsken *et al.*, 2001b). The biomass concentration represents a key parameter for the description of whole-cell processes having a direct influence on the determination of process parameters such as specific substrate uptake, product formation, or growth rates (Sonnleitner *et al.*, 1992). Gravimetrical determination of CDW, counting of colony forming units, or determination of total protein content do not allow monitoring of biomass concentration ''on-line'' or ''at-line'' during biocatalysis, which is critical for process regulation. To circumvent these problems, cell growth is often followed by measuring the dissolved oxygen tension in the cell broth or the carbon dioxide/oxygen content in the off-gas (Hüsken *et al.*, 2001b;

Neumann *et al.*, 2005; Wierckx *et al.*, 2005). However, these parameters might be influenced by stress responses of the bacteria, especially when toxic reactants are involved. In addition, an increased surface tension, foam formation, or high viscosity of the emulsion may influence analytical instruments. Furthermore, the extraction solvent should be chemically, biologically, and thermally stable, available in bulk amounts at low prices, and being nonhazardous for both operator and environment (Bruce and Daugulis, 1991; Daugulis, 1988).

In general, the evaluation of the suitability of second liquid phases by comparing partition coefficients simplifies the selection of an appropriate organic solvent with respect to biocompatibility and extractive properties. Computer-assisted screening methods were established that can help to identify the optimal solvent (Kollerup and Daugulis, 1985). However, these predictions do not replace the ultimate experimental confirmation of the suitability of a solvent or a bacterial strain for a certain two-liquid-phase biotransformation setup (Neumann *et al.*, 2005; Rojas *et al.*, 2004).

B. Application of Solvent-Tolerant Bacteria in Two-Liquid-Phase Biotransformations

In two-liquid-phase biotransformations, the extraction properties of organic solvents like toluene or octanol are favorable for the production of chemicals with intermediate polarity such as catechols (Section VI. A). However, ''normal'' bacteria are toxified by such organic solvents but can be substituted by extremophilic bacteria which can tolerate low-logP_{ow} solvents (de Bont, 1998).

1. *Mechanisms of Solvent Tolerance*

During the past decades, an increasing number of solvent-tolerant bacteria have been discovered (Ramos *et al.*, 2002; Sardessai and Bhosle, 2002). Recently, also archaea (Usami *et al.*, 2003, 2005) and eukaryotic microorganisms, such as yeast (Kawamoto *et al.*, 2001) or algae (McEvoy *et al.*, 2004), were reported to be tolerant to toxic organic solvents. A comparison among different bacterial species revealed that Gram-negative strains of the genera *Pseudomonas*, *Escherichia*, *Serratia*, and *Klebsiella* generally support the presence of organic solvents with lower logP_{ow} than Gram-positive strains (Inoue and Horikoshi, 1991). However, Gram-positive bacteria with solvent tolerance comparable to Gram-negative strains were recently characterized (Matsumoto *et al.*, 2002; Na *et al.*, 2005; Nielsen *et al.*, 2005). The majority of solvent-tolerant bacteria isolated so far belong to the genus

Pseudomonas. Strains of this genus have been reported to be tolerant to hexane, cyclohexane, *p*-xylene, styrene, octanol, heptanol, toluene, or dimethylphthalate (Ramos *et al.*, 2002; Sardessai and Bhosle, 2004; Segura *et al.*, 2003; Wery and de Bont, 2004; Wierckx *et al.*, 2005).

In general, Gram-negative bacteria are considered solvent tolerant if they can grow in the presence of an organic phase with a $\log P_{ow}$ below 4, representing conditions that are lethal for ''normal'' bacteria. Alternatively, solvent tolerance is evaluated via the survival of bacteria after a short-term exposure to organic solvents (Ramos *et al.*, 1995, 1998).

Molecular and physiological mechanisms of solvent tolerance were investigated and characterized best in *P. putida* strains, namely *P. putida* DOT-T1E (Ramos *et al.*, 1995) and *P. putida* S12 (Hartmans *et al.*, 1990). Tolerant strains were found to differ from sensitive strains in the ability to activate protection mechanisms in the cell envelope upon incubation with toxic solvents. Alterations of membrane lipids to make the cell envelope more rigid and active efflux of solvent molecules into the extracellular medium are the proposed microbial strategies to cope with toxic solvents (Ramos *et al.*, 2002). Mutants derived from solvent-tolerant *P. putida* DOT-T1E that are either deficient in *cis-trans* isomerases (Junker and Ramos, 1999) or solvent efflux pumps (Rojas *et al.*, 2001) confirmed the impact of the respective mechanisms by being significantly more sensitive to organic solvents. These observations indicate that both isomerase and efflux pumps are required for efficient solvent tolerance.

Cis-trans isomerases convert *cis*-unsaturated fatty acids of membrane phospholipids to the *trans*-isomers as a major short-term response to solvent exposure (Heipieper *et al.*, 1992; Holtwick *et al.*, 1997; Junker and Ramos, 1999; Weber *et al.*, 1994). Other alterations of membrane composition, such as changes in the degree of saturation of fatty acids, modifications of phospholipid head groups, and an increase in the total amount of phospholipids, were also observed as a result to solvent exposure (Pinkart and White, 1997; Ramos *et al.*, 1997; Segura *et al.*, 2004; Weber and de Bont, 1996). The resulting, more compact packing of the altered phospholipids in the lipid bilayer increases the rigidity of the cell envelope, thus compensating for the increased membrane fluidity caused by solvents (Heipieper *et al.*, 2003). *Cis-trans* isomerization is an urgent response to solvents, which is substituted by other, long-term mechanisms after cells have adapted to the solvents (Neumann *et al.*, 2005). Genes for *cis-trans* isomerases were identified on genomes of various pseudomonads also of solvent-sensitive strains. The *cis-trans* isomerase of solvent-sensitive *P. putida* KT2440 showed 99.5% and 95.6% amino acid sequence identity to the corresponding

enzymes of solvent-tolerant *P. putida* DOT-T1E and *P. putida* P8, respectively (Junker and Ramos, 1999). In addition, *cis* to *trans* isomerization activity was also reported in *P. putida* MW1200 and *P. putida* GPo1, which are not solvent-tolerant (Chen *et al.*, 1995b; Pinkart *et al.*, 1996), and changes of membrane lipid composition not only was observed after solvent exposure, but also occurred as a response to many environmental and physico-chemical factors like temperature, chemicals, ions, pressure, nutrients, and growth phase (Denich *et al.*, 2003; Heipieper *et al.*, 1996; Ramos *et al.*, 2001). Obviously, solvent tolerance is only one of a variety of physiological functions of *cis-trans* isomerases.

Solvent efflux pumps belonging to the resistance nodulation division family (Murakami and Yamaguchi, 2003) extrude organic solvent molecules from the inner membrane into the extracellular space (Kieboom *et al.*, 1998a,b; Ramos *et al.*, 1998; Rojas *et al.*, 2001). Such translocases are proposed to span both the inner and the outer membranes (Koronakis *et al.*, 2000; Murakami *et al.*, 2002) and act in an energy-dependent way (coupled to the proton motive force) as a long-term response to incubation with organic solvents. For adapted *P. putida* S12, the measured influx of (radioactively labeled) toluene was twofold lower than in nonadapted cells (not preincubated with sublethal concentrations of solvents) or in the presence of solvent pump inhibitors leading to energy uncoupling (Isken and de Bont, 1996). In nonadapted as compared to adapted *P. putida* S12 and *P. putida* DOT-T1E, toluene accumulated to two- and fivefold higher amounts, respectively, presumably mainly in the cell membranes (Isken and de Bont, 1996; Ramos *et al.*, 1997). It can be speculated that solvent efflux pumps, in addition to their solvent pumping activity, contribute to solvent tolerance by stabilizing the membrane structure (analogous to the structural role of porin proteins; Woodruff and Hancock, 1989). This combined effect would explain the hypersensitivity of the efflux pump knockout mutants to toluene and emphasize the important role of efflux pumps in long-term solvent tolerance (Rojas *et al.*, 2001).

As it can be inferred from the inducibility of efflux pump activity, adaptation seems to be an important prerequisite for a strain to develop a solvent tolerance phenotype. Solvent-tolerant *P. putida* strains cultivated in the presence of sublethal concentrations of organic solvents (Heipieper and de Bont, 1994; Neumann *et al.*, 2005; Ramos *et al.*, 1997, 1998) or acetic acid (Weber *et al.*, 1993) show high viabilities and relatively short growth lag phases upon incubation with a second liquid phase of toxic solvents, whereas only a fraction of cells

survive such a shock, by, for example, toluene, without preexposure (Duque *et al.*, 2001; Junker and Ramos, 1999). Yet, the genes of *cis-trans* isomerase and of the most important solvent efflux pump were shown to be expressed constitutively in *P. putida* DOT-T1E (Guazzaroni *et al.*, 2004, 2005; Junker and Ramos, 1999; Rojas *et al.*, 2003). The apparent need for adaptation in order to achieve effective solvent tolerance suggests a regulation by (still unknown) global factors that are activated upon incubation of the cells with organic solvents (Duque *et al.*, 2001; Pedrotta and Witholt, 1999; Ramos *et al.*, 2002).

In general, it is difficult to estimate the actual contributions of *cis-trans* isomerase, solvent efflux pumps, and other possible mechanisms to solvent tolerance, which ultimately enable the bacteria to grow in the presence of toxic organic solvents.

2. *Suitability of Solvent-Tolerant Strains for Industrial Applications*

What are the implications of the described solvent tolerance phenotype for the use of solvent-tolerant bacteria in two-liquid-phase biotransformations? *P. putida* MC2 was used for the production of 3-methylcatechol from glucose in the presence of a second phase of octanol (Hüsken *et al.*, 2001b). The process showed a long production lag phase of 40 hours and about 20 hours when mineral medium and LB complex medium were used, respectively. Although increasing the concentrations of the LB medium components did not improve 3-methylcatechol production, an increased energy demand for *de novo* syntheses of complex metabolic precursors could explain the extension of the production lag phase in mineral medium. However, the use of complex media can complicate downstream processing due to the presence of high concentrations of undefined medium components. Mineral media generally contain only a small number of defined components and, in contrast to complex media, allow cultivation to high cell densities, which is a prerequisite to obtain high volumetric biotransformation rates (Riesenberg, 1991).

The presence of toluene decreased the biomass yield and increased the maintenance energy demands of *P. putida* S12 (Isken *et al.*, 1999). Proteome analysis of *P. putida* DOT-T1E revealed an upregulation of proteins involved in energy metabolism as a consequence of incubation with toluene (Segura *et al.*, 2005). *P. putida* DOT-T1E cells are able to grow in the presence of a second liquid phase of decanol or nonanol without significant growth inhibition after preexposure to sublethal concentrations of organic solvents (Neumann *et al.*, 2005; Rojas *et al.*, 2004). However, growth is completely inhibited in the presence of a second octanol phase, although adapted cells survived

for at least 24 hours. Octanol accumulates to higher maximum membrane concentrations (588 mM) as compared to decanol (379 mM) (Neumann *et al.*, 2005). Apparently, energy generation of *P. putida* DOT-T1E was sufficient to reduce decanol, nonanol, or octanol concentrations in the membranes below lethal levels. The increased energy demand for the extrusion of octanol, as compared to decanol and nonanol extrusion, however, did not allow cell growth. An engineered strain of *P. putida* S12 used for the production of phenol from glucose was able to grow in the presence of a second phase of octanol during fed-batch cultivation under nitrogen limited conditions, suggesting a higher level of energy metabolism of *P. putida* S12 as compared to *P. putida* DOT-T1E (Wierckx *et al.*, 2005).

Although stable growth in the presence of a second phase of octanol or toluene can be achieved by the refinement of adaptation and cultivation procedures, these observations indicate that the high energy demand for solvent tolerance may limit or prevent (energy dependent) biocatalysis. Indeed, production rates of two-liquid-phase biotransformation processes with octanol as a second phase are rather low. For example, volumetric product formation rates (based on the totals of product concentration, working volume, and biotransformation time) for 3-methylcatechol synthesis are 0.054 and 0.012 g $liter^{-1}$ $hour^{-1}$ with toluene (Hüsken *et al.*, 2001b) and *m*-xylene (Rojas *et al.*, 2004) as the starting compounds, respectively, and 0.008 g $liter^{-1}$ $hour^{-1}$ for the synthesis of phenol from glucose (Wierckx *et al.*, 2005). Here, the implementation of membranes to decrease phase toxicity or mathematical models may be useful to improve such processes (Hüsken *et al.*, 2002, 2003).

Heterologous expression of genes encoding solvent tolerance mechanisms in host strains that provide an increased metabolic activity might be a promising approach to generate competitive solvent-tolerant biocatalysts. Broad host range plasmids have been constructed encoding genes of *cis-trans* isomerase from solvent-tolerant *P. putida* P8 (Holtwick *et al.*, 1997) or *P. putida* DOT-T1E (Junker and Ramos, 1999) or solvent efflux pump genes from *P. putida* S12 (Kieboom *et al.*, 1998a). Thereby, cells of a solvent-sensitive *P. putida* strain overproducing a recombinant solvent efflux pump were able to tolerate higher toluene concentrations than cells of the parent strain (Kieboom *et al.*, 1998a). A *cis-trans* isomerase knockout mutant of *P. putida* DOT-T1E incubated with sublethal concentrations of toluene provided a longer growth-lag phase than the complemented mutant overproducing recombinant *cis-trans* isomerase (Junker and Ramos, 1999). Such constructs may as well be applicable to bacterial strains different

from *Pseudomonas*. This was shown by the overproduction of *cis-trans* isomerase in *E. coli*, which enabled the *E. coli* strain to synthesize *trans*-unsaturated fatty acids (Holtwick *et al.*, 1997). The high level of energy metabolism of α-proteobacteria (Section IV) might enable both stable solvent tolerance and the supply of sufficient reducing equivalents for oxidative biocatalysis.

To be feasible for industrial implementation, bioprocesses require reproducible cell growth and high productivities. Unproductive time periods due to adaptation and cell growth inhibition still make biotransformations based on solvent-tolerant *P. putida* strains and octanol or toluene as second phases rather unattractive. Decanol was proposed to be a useful second liquid phase in biotransformations with *P. putida* DOT-T1E since it significantly increased the overall volumetric 3-methylcatechol accumulation rate (0.134 g liter^{-1} hour^{-1}) as compared to the rate achieved with octanol as a second phase (0.012 g liter^{-1} hour^{-1}) and allowed cell growth (Neumann *et al.*, 2005; Rojas *et al.*, 2004). However, also *E. coli* JM101 and *E. coli* W3110 grow in mineral medium containing a second phase of decanol ($\log P_{ow}$, 4.0; D. Meyer and A. Schmid, unpublished data) or heptane ($\log P_{ow}$, 4.0; Favre-Bulle *et al.*, 1991) without prior adaptation. The saturation ratio of fatty acids in *E. coli* membranes did not change significantly in the presence of decanol or nonanol, which underlines that such organic solvents also can be tolerated by solvent-sensitive microorganisms such as *E. coli* (Sullivan *et al.*, 1979).

Overall, no clear benefit of the use of solvent-tolerant *P. putida* for redox biocatalysis has been found to date. Apparently, the solvent-tolerance potential of *P. putida* strains cannot yet be fully exploited for economical two-liquid-phase bioprocesses based on organic solvents that are not tolerated by ''normal'' bacteria.

VII. Conclusions and Future Prospects

Metabolically active cells are a prerequisite for efficient redox biocatalysis, since they enable the synthesis of high concentrations of functional enzymes, cofactor regeneration, and extrusion of toxic chemicals by solvent efflux pumps in solvent-tolerant bacteria. Strains providing a higher NAD(P)H regeneration capacity than *E. coli* are interesting candidates as hosts for NAD(P)H-dependent biotransformations. However, high respiration activity may limit the availability of oxygen and NAD(P)H for oxygenase-based biocatalysis. Oxygen transfer might be increased by improving the reactor design or by reaction engineering, for example, via the addition of hydrogen peroxide and catalase to the cell broth.

Enzymatic background activity of metabolically active cells can catalyze the degradation of substrates and products as well as side-product formation. The use of bacterial strains that provide a defined metabolic background compatible with the desired biotransformation is, therefore, favorable but might require preliminary screening and characterization of new or metabolic engineering of known strains. More sophisticated tools for heterologous gene expression in strains different from *E. coli* are required in order to use recombinants based on exotic bacterial hosts as biocatalysts in a routine way.

A second phase of an organic solvent in two-liquid-phase biotransformation allows the production of high amounts of toxic products, stabilizes reactants, and increases oxygen transfer. However, the selection of a suitable organic solvent is not trivial and depends on parameters like biocompatibility, extraction properties, and practical handling. The use of solvent-tolerant bacteria in two-liquid-phase biotransformations with organic solvents of intermediate polarity, such as toluene, may allow the synthesis of a broader range of products. However, critical factors, such as productivity lag phases, growth inhibition, and enduring adaptation procedures, have to be carefully evaluated in order to asses the suitability of solvent-tolerant bacteria for industrial applications.

This chapter elucidates the importance to improve and intensify a process by engineering efforts on the molecular, physiological, and reaction levels. Advances in the field of microbiology will provide an increasing number of enzymes and microorganisms potentially useful for redox biocatalysis. The use of host bacteria optimized for both the catalysis of the desired reaction and the applicability in an appropriate process setup will further improve the productivity of redox biocatalysis.

Acknowledgments

The authors are indebted to Dr Michael Schedel (Bayer HealthCare AG, Wuppertal, Germany) and to Dr Lars Blank (University of Dortmund, Germany) for fruitful discussions.

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The Biosynthesis of Polyketide Metabolites by Dinoflagellates

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I. Introduction

Flagellated species of algae account for 90% of harmful algal blooms (HAB), and in this group approximately 75% are dinoflagellates (Smayda, 1997). More importantly, the geographic expansion of HAB outbreaks has been attributed to 10–12 species of these toxic dinoflagellates (Hallegraeff, 1993). Dinoflagellates are members of the eukaryotic subgroup *Alveolates*, along with apicomplexans and ciliates. They are biflagellate protists (unicellular eukaryotes) that possess numerous eukaryotic traits with some irregularities (Table I). Some dinoflagellates produce secondary metabolites of unparalleled complexity. Some may have therapeutic potential, but many are highly toxic.

Marine algal toxins have been grouped based on the six human illnesses: azaspiracid poisoning (AZP), amnesic shellfish poisoning (ASP), ciguatera fish poisoning (CFP), diarrheic shellfish poisoning (DSP), neurotoxic shellfish poisoning (NSP), and paralytic shellfish poisoning (PSP). Table II summarizes the illnesses, their sources, poisoning symptoms, and causative toxins (Fig. 1). Four of the six illnesses

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 59 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(06)59004-5

TABLE I

SHARED CHARACTERISTICS OF MOST DINOFLAGELLATES

Characteristic	References
Relatively large amount of DNA	Spector, 1984
Unusual sterols	Withers, 1983
Hypermethylated DNA	Ten Lohuis and Miller, 1998
Hydroxymethyluracil substituted for thymine	Rae, 1976
Most are uninucleates, but some binucleates exist	Rizzo, 2003
Permanently condensed chromosomes	Rizzo, 2003
Lack histones and nucleosomes	Moreno Diaz *et al.*, 2005
Possess a vegetative haploid nuclear phase	Santos and Coffroth, 2003
Typical eukaryotic nuclear envelope, but it remains intact during mitosis	Bhaud *et al.*, 2000
Typical eukaryotic 9 + 2 axoneme flagella	Maruyama, 1985
Membrane-bound organelles (endoplasmic reticula, Golgi apparatus, mitochondria, and chloroplasts)	Spector, 1984
Percentages of repetitive DNA (50–60%) consistent with higher eukaryotic genomes	Moreno Diaz *et al.*, 2005
Typical eukaryotic cell cycle	Moreno Diaz *et al.*, 2005
Heavy (28S) and light (17S) rRNA that is structurally similar to higher eukaryotes	Herzog and Maroteaux, 1986
Lack the TATA-box (typical eukaryotic promoter)	Guillebault *et al.*, 2002

are caused by dinoflagellate-derived polyketide toxins. The two exceptions are domoic acid, a kainic acid analog produced by diatoms in the *Pseudo-nitschia* genus (Ramsey *et al.*, 1998), and the saxitoxins, a group of cyclic perhydropurine compounds produced by *Moraxella* (an *Alexandrium tamarenses* intracellular bacteria) (Kodama *et al.*, 1990), cyanobacteria (Ferreira *et al.*, 2001), and species of *Alexandrium* (Hold *et al.*, 2001).

The majority of dinoflagellate toxins are polyketide in origin. Polyketides are biosynthesized via the sequential Claisen condensations of small carboxylic acid subunits in a fashion reminiscent of fatty acid

TABLE II

ALGAL-RELATED HUMAN POISONING SYNDROMES AND CAUSATIVE AGENTS

Syndrome	Toxins	Symptoms	Causative organisms	Route of acquisition
Azaspiracid poisoning (AZP)	Azaspiracids	Acute gastroenteritis	*Protoperidinium crassipes*	Eating tainted shellfish
Amnesic shellfish poisoning (ASP)	Domoic acid	Gastroenteritis followed by neurologic manifestations leading to amnesia, coma, and death in severe cases	*Pseudo-nitschia* multiseries;	Eating tainted shellfish
Ciguatera fish poisoning (CFP)	Ciguatoxin, maitotoxin	Acute gastroenteritis followed by paresthesias and other neurologic symptoms	*Gambierdiscus toxicus*	Eating predacious reef fish (e.g., barracuda)
Diarrheic shellfish poisoning (DSP)	Okadaic acid dinophysistoxins	Acute gastroenteritis	*Prorocentrum lima* *Dinophysis acuta* *D. fortii* *D. norvegica*	Eating tainted shellfish
Neurotoxic shellfish poisoning (NSP)	Brevetoxins	Neurologic symptoms	*Karenia brevis* *K.* cf *brevis* (New Zealand) Raphidophytes	Eating tainted shellfish
Paralytic shellfish poisoning (PSP)	Saxitoxins	Acute paresthesias and other neurologic manifestations; may progress rapidly to respiratory paralysis	*Alexandrium catenella* *A. minutum* *A. tamarense* *Gymnodinium catenatum* *Pyrodinium bahamense*	Eating tainted shellfish

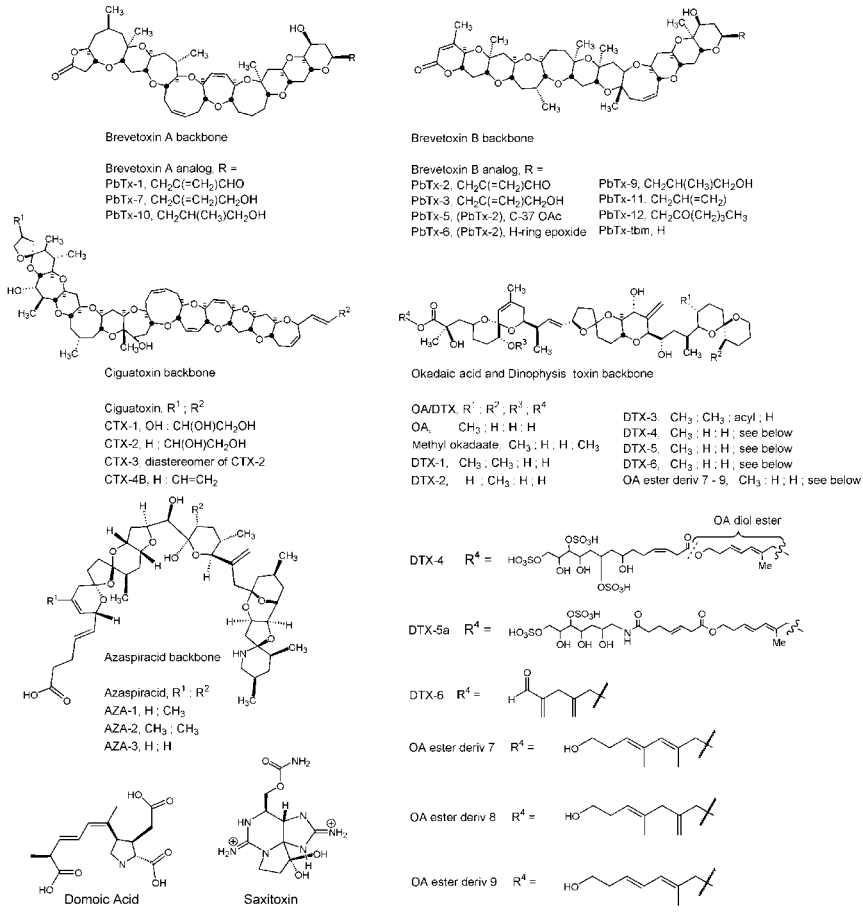


FIG. 1. Marine algal toxins responsible for classified human illnesses. Also shown are ester derivatives of okadaic acid.

biosynthesis. Polyketide synthases (PKSs) have been traditionally classified into three types. However, many variants have recently emerged that do not fit this classification scheme, leading some to suggest that it should be abandoned entirely (Muller, 2004; Shen, 2003). Based on their size, functionalities, and complex structures, one would predict that dinoflagellate-derived polyketides are biosynthesized by Type I modular PKSs. These enzymes are composed of multiple enzymatic domains on the same polypeptide chain. Thus, they are large,

multifunctional, and can generate complex structures. Typical structures produced by Type I PKSs are polyenes, macrocycles (including macrolides and nonmacrolides), and polyethers, whereas Type II PKSs produce aromatic polyketides, in general.

Our knowledge of polyketide biosynthesis in dinoflagellates is derived entirely from stable isotope incorporation experiments, which have confirmed the head-to-tail assembly of acetate units with some irregularities. To date, not a single gene has been linked to dinoflagellate polyketide biosynthesis. However, PKS genes have been localized by fluorescence *in situ* hybridization (FISH) to the toxic dinoflagellate *Karenia brevis* (Snyder *et al.*, 2005). This is the first indication that dinoflagellates carry their own suite of resident PKS genes. Additionally, PKS-encoding genes have been identified in a dinoflagellate expressed sequence tags (ESTs) library (*vide infra*) (Lidie *et al.*, 2005). The motivation to identify genes related to polyketide biosynthesis in dinoflagellates is twofold. First, the manipulation of PKS associate genes could provide unprecedented biosynthetic capability to the growing field of combinatorial biosynthesis (Rodriguez and McDaniel, 2001). Second, an understanding of regulation of polyketide biosynthetic genes may present novel and improved strategies for predicting and monitoring toxic blooms.

Molecular genetic studies of dinoflagellates are very limited. While a survey of Genbank for dinoflagellate genes returns more than 20,000, most of them are related to photosynthesis, protein synthesis (i.e., rRNA), or located in the chloroplast or mitochondrial genomes, or are from EST libraries. Very little has been done in terms of finding a targeted gene from a dinoflagellate nuclear genome. There are many obstacles that will need to be addressed before these types of studies of dinoflagellates become more commonplace. These obstacles have been reviewed in the past (Plumley, 1997) and will be briefly mentioned here in Section IV. Also included, is a survey of strategies that others have used for the study of functional genes in dinoflagellates that may, in the future, be applied to dinoflagellate polyketide toxin biosynthesis.

II. Recently Discovered Dinoflagellate Polyketides

Numerous dinoflagellate polyketide variants have been identified over the past 5 years. However, this section is not meant to be an exhaustive review of new structures. The reader is directed to the many excellent reviews of dinoflagellate toxins for a more comprehensive account (Balmer-Hanchey *et al.*, 2003; Holmes and Lewis, 2002; Kobayashi and Tsuda, 2004; Kobayashi *et al.*, 2003; Rein and Borrone, 1999; Shimizu, 2003). We have chosen to focus on new toxins identified since our last

review of the field (Rein and Borrone, 1999), as well as structural refinements to known compounds. This section is further limited to polyketides isolated either from laboratory cultures of dinoflagellates or from wild samples or plankton tows. Many analogs of previously identified dinoflagellate-derived polyketides have been isolated from shellfish or marine invertebrates. However, it is not clear that these analogs are produced *de novo* by the dinoflagellates themselves or if they are shellfish metabolites. Admittedly, it is not even certain that some of the variants isolated from laboratory cultures of dinoflagellates are not bacterial metabolites, as most of the dinoflagellates in question have not been grown axenically. We will also not cover the pharmacology of marine toxins as this is beyond the scope of this review and would be best covered separately. Again, the reader is directed to recent reviews on the subject (Rein and Borrone, 1999; Yasumoto, 2001).

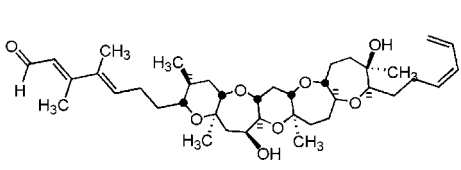
Only a handful of truly novel dinoflagellate polyketides, with respect to backbone structure, have been discovered since we last reviewed this field. The vast majority of new polyketides identified are structural variants of known compounds. Novel structures include azaspiracid, brevenal, prorocentin, and a number of new amphidinolides.

A. From *Protoperidinium crassipes*

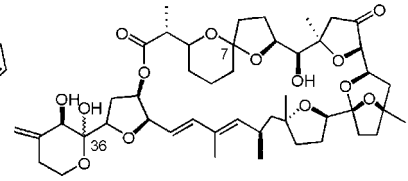
In 1995, several people in the Netherlands became ill after eating mussels, which had been farmed in Killarney Harbor in Ireland. The causative toxin, named azaspiracid, was first isolated from 20 kg of mussel tissue (Satake *et al.*, 1998). It was later found in isolated cells of what had previously been believed to be a nontoxic dinoflagellate, *Protoperidinium crassipes.* Two hundred cells were hand picked from plankton samples for LC-MS analysis. Two structural analogs, AZA2 and AZA3, the 8-methyl and 22-desmethyl analogs respectively (Fig. 1) (James *et al.*, 2003a; Ofuji *et al.*, 1999), were also associated with this dinoflagellate. Numerous hydroxyl derivatives have also been found, but all from shellfish (James *et al.*, 2003b). After the total synthesis of azaspiracid-1 was completed by the Nicoloau group, the structure was revised to that shown in Fig. 1 (Nicolaou *et al.*, 2004).

B. From *Karenia brevis*

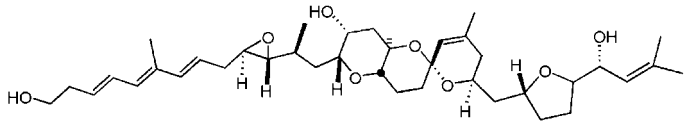
Brevenal has been isolated from cultures of *Karenia brevis*, the Florida red tide organism (Fig. 2) (Bourdelais *et al.*, 2005). It is roughly half the size of the brevetoxins, having only 4 fused ether rings compared to 10 for brevetoxin A and 11 for brevetoxin B. The structure



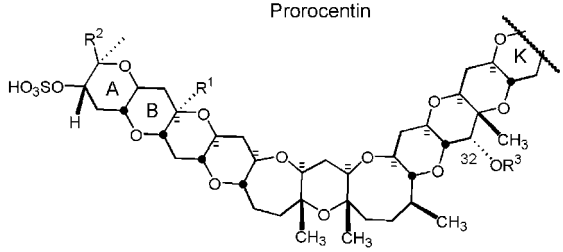
Brevenal



Pectenotoxin-12



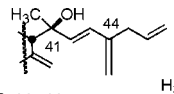
Prorocentin



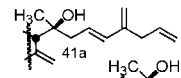
Yessotoxin backbone

YTX, R^1 ; R^2 ; R^3 ; K ring substituent

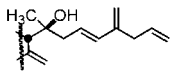
YTX, H ; $CH_2CH_2OSO_3H$; H ;



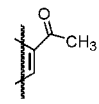
41a-homoYTX, H ; $CH_2CH_2OSO_3H$; H ;



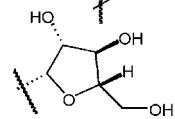
9-Me-41a-homoYTX, CH_3 ; $CH(OH)CH_2OH$; H ;



1,3-enone isomer-YTX, H ; $CH_2CH_2OSO_3H$; H ;



Glycoyessotoxin A, H ; $CH_2CH_2OSO_3H$;



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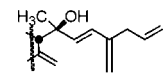


FIG. 2. Novel polyketides from dinoflagellates. See text for details.

of brevenal is distinct from, but somewhat reminiscent of, the hemibrevetoxins that were reported by Shimizu in 1989 (Prasad and Shimizu, 1989; Shimizu *et al.*, 1989). Brevenal appears not to share the sodium channel activity of the brevetoxins, but surprisingly acts as a brevetoxin antagonist (Bourdelais *et al.*, 2004). Several new side-chain variants of brevetoxin B have been isolated from *K. brevis* (Baden *et al.*, 2005). These include PbTx-11 (propenyl), PbTx-12 (2-oxo-hexyl), and PbTx-tbm (no side chain) (Fig. 1). Also reported were the dimethyl acetal and hemiacetal of PbTx-2. However, these are more likely to be artifacts of the isolation process, which includes a chromatography step on silica gel with acetic acid and methanol mobile phase, rather than toxin variants produced by the dinoflagellate.

C. From *Prorocentrum* and *Dinophysis*

Prorocentin (Fig. 2) was isolated from cultures of the dinoflagellate *Prorocentrum lima* (Lu *et al.*, 2005). The C/D spiroketal fused ring system of prorocentin is highly reminiscent of the A/B ring system of okadaic acid (OA). Because it was isolated from an OA-producing strain of *P. lima*, the authors suggested that the two polyketides share a common biogenic pathway.

In addition to prorocentin, a number of variants of OA and dinophysis toxins have been identified. Several new ester derivatives of OA have been described (Fig. 1) (Fernandez *et al.*, 2003; Suarez-Gomez *et al.*, 2001, 2005). In addition, two isomers of OA, DTX-2b, and -2c, have been observed in HPLC-MS analysis of phytoplankton extracts (Draisci *et al.*, 1998). These compounds have the same molecular mass and fragmentation pattern as OA, yet have different HPLC retention times. The authors suggest that these are spiroketal epimers of OA.

Two isomers of pectenotoxin, PTX-12 (36R and 36S) having an *exo* methylene at the terminal pyran ring as opposed to a methyl group, were isolated from *Dinophysis acuta*, *D. acuminata*, and *D. norvegica* (Fig. 2) (Miles *et al.*, 2004c). It had been believed that the hydrolysis of the PTXs was an enzymatic process in the digestive glands of shellfish; however PTX-2-seco acid and 7-epi-PTX-2-seco acid were also isolated from *Dinophysis acuta* (James *et al.*, 1999).

D. From *Protoceratium reticulatum*

Three glycosylated derivatives of homoyessotoxins were isolated from culture media of *Protoceratium reticulatum.* These compounds possessed mono-, di-, and tri-β-arabinofuranose moieties at the 32-hydroxyl

group. They were named protoceratin 3, 2, and 4 respectively (Konishi *et al.*, 2004). These represent the first example of glycosylated polyether ladder toxins from a dinoflagellate, however, the polyether ladder prymnesins from haptophyte *Prymnesium parvum* are also glycosidic (Igarashi *et al.*, 1999). Later, the corresponding mono arabinofuranose derivative of yessotoxin, glycoyessotoxin A, was isolated from cell mass of *Protoceratium reticulatum* cultures (Fig. 2) (Souto *et al.*, 2005).

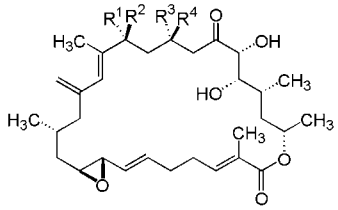
As part of a program to evaluate yessotoxin profiles in shellfish and phytoplankton and to evaluate the pharmacology of analogs, several new analogs were identified from shellfish and from cultures of *Protoceratium reticulatum*. At least seven new YTX analogs were isolated and characterized from *P. reticulatum* (Fig. 2). These include, 41a-homoyessotoxin, 9-methyl-41a-homoyessotoxin, nor-ring A-yessotoxin (not shown) (Miles *et al.*, 2004b), a 1, 3-enone isomer of heptanor-41-oxo-YTX (Miles *et al.*, 2004a), (44-*R,S*)-44, 55-dihydroxyyessotoxin (Finch *et al.*, 2005), and the first examples of YTXs having an amide side chain (not shown) (Miles *et al.*, 2005).

E. From *Amphidinium* sp.

Symbiotic dinoflagellates of the genus *Amphidinium* continue to be a rich source of polyketides (Fig. 3). The amphidinolides are an expanding group of cytotoxic macrolides (Kobayashi and Tsuda, 2004). Two variants of amphidinolide B (B4 and B5) were reported in 2005 (Tsuda *et al.*, 2005). Two other amphidinolide series share the same carbon skeleton as the amphidinolide B series. The G and H series differ from the B series in that they have a C26 hydroxyl. The B and H series are lactonized at the C25 hydroxyl, whereas the G-series is lactonized at the C26-hydroxyl. New amphidinolides in these series include amphidinolide G2, G3, H2, H3, H4, and H5 (Kobayashi *et al.*, 2002). Within these three series, variations are seen in the configurations at C16, C18, and C22, and the level of saturation of the C6–C7 bond.

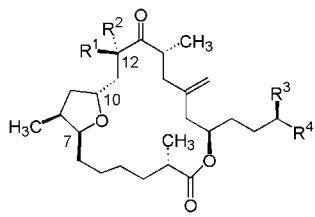
The amphidinolide T series was first reported in 2000 (Tsuda *et al.*, 2000). Since then, four additional members were discovered (T2–T5) (Kobayashi *et al.*, 2001; Kubota *et al.*, 2001a). All are 19-membered ring macrolides possessing a furan ring that spans C7–C10. This series varies in the oxidation state and configuration at C12. Additionally, amphidinolide T2 has an extra carbon and hydroxyl group on its side chain.

A number of novel amphidinolides, U, V, W, X, and Y were recently described (Figs. 3 and 5) (Kubota *et al.*, 2000a; Shimbo *et al.*, 2002; Tsuda *et al.*, 1999, 2003a,b). While these structures are unique, some do share



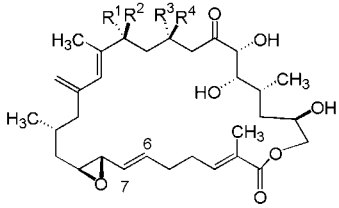
B, R^1 ; R^2 ; R^3 ; R^4

B, OH ; CH_3 ; OH ; H
B4, H ; CH_3 ; OH ; H
B5, CH_3 ; H ; H ; OH



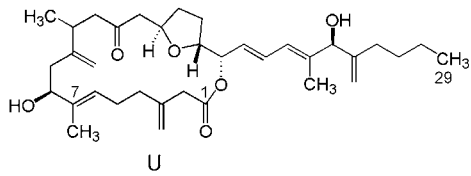
T, R^1 ; R^2 ; R^3 ; R^4

T2, OH ; H ; OH ; CH_3
T3, OH ; H ; H ; H
T4, H ; OH ; H ; H

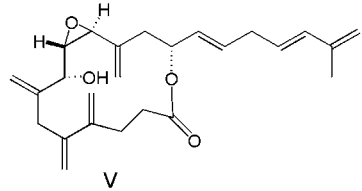


G, R^1 ; R^2 ; R^3 ; R^4

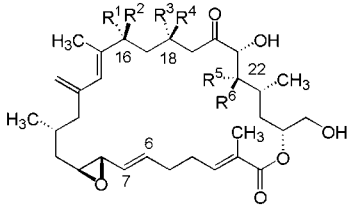
G, H ; CH_3 ; OH ; H
G2, CH_3 ; H ; H ; OH
G3, 6,7 dihydro of G



U

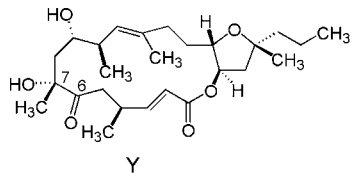


V



H, R^1 ; R^2 ; R^3 ; R^4 ; R^5 ; R^6

H, H ; CH_3 ; OH ; H ; OH ; H
H2, CH_3 ; H ; H ; OH ; OH ; H
H3, H ; CH_3 ; H ; OH ; OH ; H
H4, H ; CH_3 ; H ; OH ; H ; OH
H5, 6,7 dihydro of H2



Y

FIG. 3. New amphidinolides from *Amphidinium* species.

similarities with previously characterized amphidinolides. For instance, C7 through C29 and C1–C8 of amphidinolide U correspond to C12–C34 and C1–C8 of amphidinolides C and A, respectively. C9–C16 of amphidinolide W corresponds to C6–C13 of amphidinolide H. These similarities suggest a common biogenic origin for several of the amphidinolide series. Amphidinolides X and Y are entirely unique but related to one another. The oxidative cleavage of the C6–C7 bond of amphidinolide Y with lead tetraacetate yields amphidinolide X. This suggests that amphidinolide Y is certainly a biogenic precursor to amphidinolide X.

Other notable developments within the amphidinolides include the isolation of an acetylated version of amphidinolide C (C2) (Kubota *et al.*, 2004), the total syntheses of amphidinolides A (Trost and Harrington, 2004) and W (Ghosh and Gong, 2004), which led to the revision of these structures, and the determination of the absolute configuration of amphidinolides G and H (Kobayashi *et al.*, 2000).

In addition to cytotoxic macrolides, dinoflagellates of the genus *Amphidinium* produce several classes of polyhydroxy polyenes. These include the amphidinols, luteophanols, lingshuiol, and colopsinols. The amphidinols, luteophanols, and lingshuiol are closely related with two pyran rings in the central portion of the chain, giving them a hairpinlike conformation, which is believed to contribute to their membrane disrupting activities (Echigoya *et al.*, 2005). Currently, there are seven known amphidinols. They are identical in the central portion of the molecules and vary in the level of saturation, oxidation, and substitution of the side chains (Echigoya *et al.*, 2005). Amphidinol 7 is unique among them in that it possesses a truncated (by 10 carbons over AM 1) polyene chain (Fig. 4) (Morsy *et al.*, 2005). The absolute configuration of AM 3 was established by analysis of C–H spin coupling constants (Murata *et al.*, 1999). The polyhydroxylated lingshuiol A and B were isolated from an epiphytic species of *Amphidinium* (Huang *et al.*, 2004). These are identical to the previously isolated amphidinol 2 from C29–C65 except that amphidinol 2 is saturated from C58–C61. The C1–C28 portion of lingshuiol is distinct from amphidinol 2. Finally, colopsinols A–D are similar to the amphidinols except that they have a single pyran ring at the terminus of the polyketide chain (Fig. 4) (Kubota *et al.*, 1999, 2000b). The colopsinols vary in the extent of glycosylation.

F. Other

Like many dinoflagellate toxins, spirolides were first isolated from shellfish in 1995 and presumed to be of dinoflagellate origin on the basis of their structures (Fig. 4). In 2001, the source of these compounds

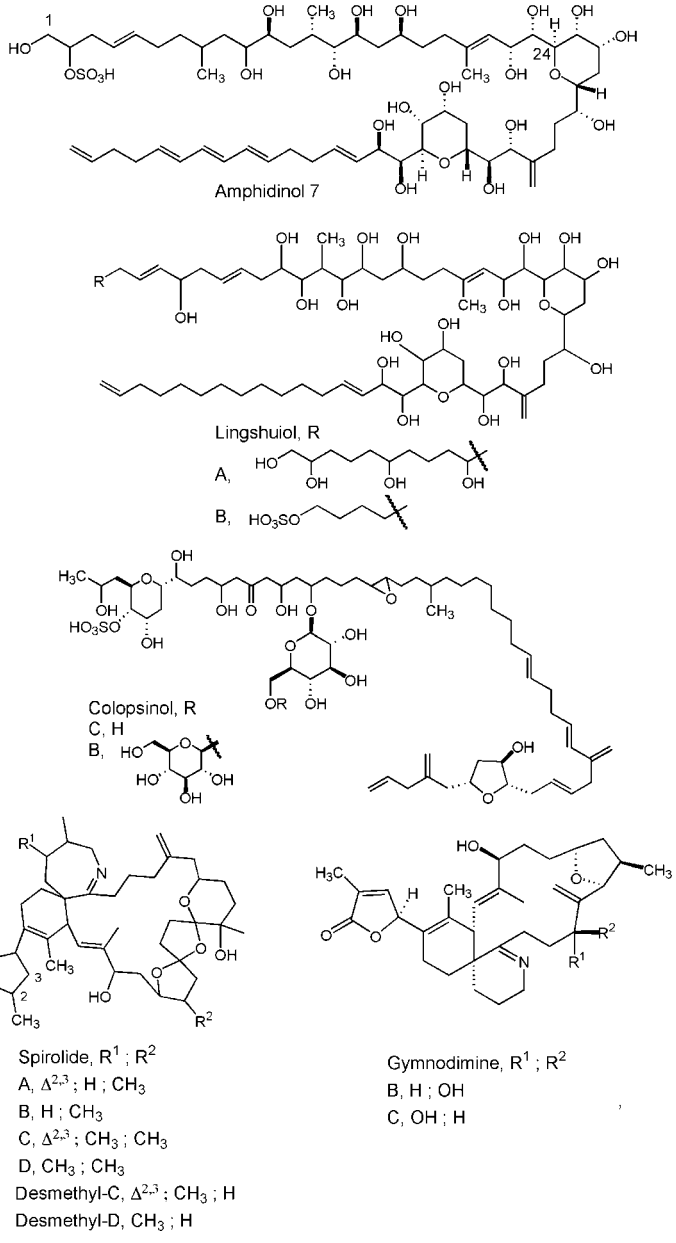


FIG. 4. Amphidinol 7, lingshuiol, colopsinols B and C, spirolides A–D, and gymnodimine B and C.

was identified as *Alexandrium ostenfeldii* (Cembella *et al.*, 1999). Since then, spirolides A and C, and 13-desmethyl C and D have been characterized from this organism (Hu *et al.*, 2001; Sleno *et al.*, 2004). The relative configurations of the stereocenters of the spirolides were established in 2001 by extensive ROESY and NOESY NMR analysis (Falk *et al.*, 2001). New gymnodimine isomers, gymnodimine B and C, were isolated from *Karenia selliformis* (Fig. 4) (Miles *et al.*, 2000, 2003). The absolute configuration of gambieric acid from *Gambierdiscus toxicus* was determined by a combination of Mosher analysis, NMR, and chiral HPLC (Morohashi *et al.*, 2000).

III. Stable Isotope Incorporation Experiments

The first biosynthetic studies of dinoflagellate-derived polyketides were performed on the brevetoxins (Chou and Shimizu, 1987; Lee *et al.*, 1989). The incorporation of [1-^{13}C], [2-^{13}C], and [1, 2-^{13}C] labeled acetate revealed a head-to-tail arrangement of acetate units over much of the carbon skeleton confirming the polyketide origins of these compounds. However, some unusual patterns of incorporation were observed. Most notable was the frequent deletions of C1 of acetate leaving subunits of c–m–m, c–m–m–m, and c–m–m–m(m). These patterns have been repeated in every dinoflagellate derived polyketide studied to date.

A. Amphidinolides

In terms of biosynthetic experiments, the most extensively studied group of dinoflagellate-derived polyketides are the amphidinolides. Stable isotope incorporation experiments have been performed on amphidinolide B, C, H, J, T1, W, X, and Y (Fig. 5) (Kobayashi *et al.*, 1995, 2001; Kubota *et al.*, 2001b; Sato *et al.*, 2000; Tsuda *et al.*, 2001, 2002, 2003a,b). These studies have revealed that all carbons of the amphidinolides, including pendent methyl groups, which are more typically derived from S-adenosylmethionine (SAM) or propionate incorporation, are derived from acetate. This is in contrast to the brevetoxins, where some of the pendent methyl groups were labeled with S-adenosyl methionine.

The biosynthetic subunits, which were observed in the brevetoxins, are also apparent in the amphidinolides. The c–m–m fragment is observed in amphidinolides C, J, T1, W, and X. The c–m–m–m fragment is observed in amphidinolides H and T1, and the c–m–m–m(m) fragment is observed in amphidinolide Y. However, the C1 deletions appear to be even more frequent in the amphidinolides. The frequent C1 deletions

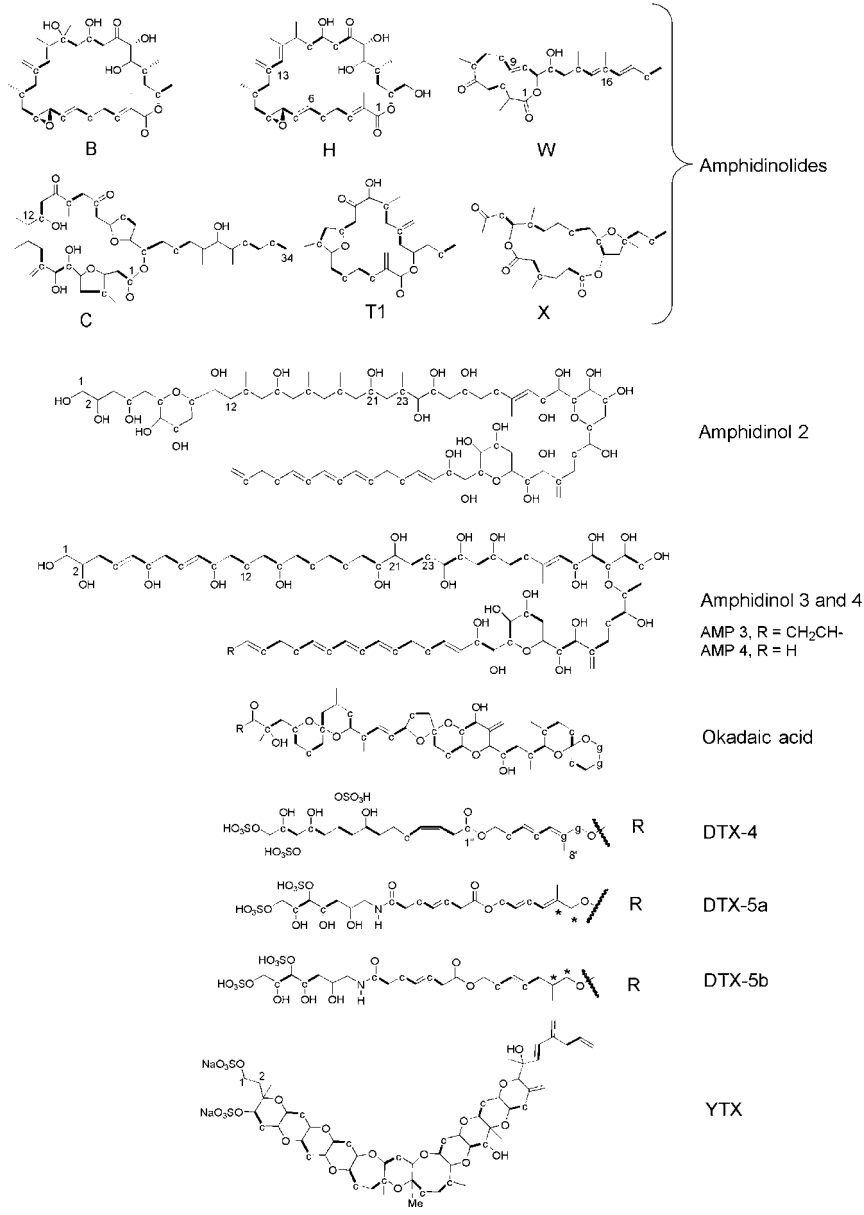


FIG. 5. Labeling pattern of polyketides from stable isotope feeding experiments including amphidinolides, amphidinols, okadaic acid and dinophysis toxins, and yessotoxin. Legend: g = glycolate, c = C1 acetate, m = C2 acetate, Me represents methyl

and incorporation of pendent methyl groups has led to the identification of a wider variety of fragments than observed in prior studies. A c–m–m (m) fragment appears in amphidinolides B, J, and Y. A c–m–m–m fragment appears in amphidinolides B. A c–m–m(m)–m(m) fragment appears in amphidinolides C and H, and a c–m–m(m)–m–m(m) fragment appears in amphidinolide C. Also noteworthy is the appearance of an m(m)–m fragment in amphidinolides B, H, T1, and W. This pattern appears at the termination of the polyketide chain of these macrolides.

The biosynthetic origin of these fragments has been the subject of speculation for many years. Shimizu and Nakanishi invoked the incorporation of citric acid cycle intermediates to account for C1 deletions in the brevetoxins (Chou and Shimizu, 1987). Incorporation of atypical subunits like succinate and ketoglutarate would require a truly novel PKS. On the other hand, Wright proposed a Favorskii-like rearrangement in the construction of OA (Wright *et al.*, 1996). Later, Rawlings (1999) suggested a Tiffeneau-Demyanov rearrangement of an α, β-epoxy ketone. A comparison of the intensity ratios (labeled/unlabeled) of the different types of acetate methyls for amphidinolide B indicate that these ratios are similar whether the carbon is incorporated as part of an intact acetate, a cleaved acetate, or as a pendent methyl or methylene at either a C1 or C2 of an acetate subunit (Tsuda *et al.*, 2001). This would seem to imply that the labeled precursors are not diluted in the metabolic pool prior to incorporation and are incorporated as intact acetate units and modified only after incorporation into the growing polyketide chain. This hypothesis is not consistent with the incorporation of citric acid cycle intermediates but is more consistent with Wright's Favorskii rearrangement hypothesis. Further support for this hypothesis is the Favorskii type rearrangement observed during the biosynthesis of enterocin and the wailupemycins by a marine bacterium, *Streptomyces maritimus* (Xiang *et al.*, 2004). In these metabolites one carbon derived from C1 of acetate is extruded from the parent polyketide chain but is retained as a pendent carboxyl group. The *encM* gene, found in the enterocin gene cluster, codes for a flavin-dependent oxygenase (EncM) and was demonstrated to be solely responsible for the Favorskii rearrangement.

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from S-adenosyl methionine, bold lines represent intact acetates, unlabeled positions represent C2 of acetate unless otherwise noted. The red subunit originated from glycine. For amphidinol 3, only C1 labeled acetate was fed as a precursor, the origin of other positions was not determined. * Glycolate feeding experiments were not conducted for these polyketides, but source is presumed to be glycolate by analogy with DTX-4.

While common in dinoflagellate-derived polyketides, the incorporation of C2 of acetate as a pendent methyl group is relatively rare among other organisms. Some bacterial polyketides have shown this same pattern. In the biosynthesis of curacin A (Chang *et al.*, 2004) and jamaicamide (Edwards *et al.*, 2004), the incorporation of C2 of acetate is performed by an HMG-CoA synthetase-like enzyme via an aldol condensation followed by a decarboxylation. The pathways for the antibiotic TA (Paitan *et al.*, 1999), mupirocin (El-Sayed *et al.*, 2003), leinamycin (Cheng *et al.*, 2003), and difficidin believed to be made by PksX (Albertini *et al.*, 1995) also contain HMG-CoA synthase-like genes. These genes show high homology to each other and lower homology to HMG-CoA synthase genes associated with terpene biosynthesis. In these examples, the growing polyketide chain is modified at a carbonyl from C1 of acetate. What is unusual about dinoflagellate-derived polyketides is the incorporation of the acetate methyl group at carbon derived from C2 of acetate. Most of the pendent methyl groups of the amphidinolides are incorporated at C1 of acetate. However, 14 of the 36 methyl groups of amphidinolide B, C, H, J, T1, W, and Y are appended to C2 of acetate. It may be noteworthy that an acetate-derived pendent methyl group never appears at C2 of an *intact* acetate unit among the amphidinolides, or any other dinoflagellate-derived polyketide. Seven pendent methyl groups are present in the brevetoxins. Four of those are at C2 of an intact acetate unit; however those four methyl groups are derived exclusively from S-adenosyl methionine. Similarly, six pendent methyl groups and one methylene are present in yessotoxin. The single methyl group that is positioned at C2 of an intact acetate unit is derived from S-adenosyl methionine (Satake, 2000).

A comparison of labeling patterns for amphidinolides B and H revealed the surprising conclusion that they do not share identical incorporation patterns even though their carbon skeletons are identical. Stable isotope incorporation experiments revealed different patterns of incorporation for carbons 16–19. For amphidinolide B, the pattern is cm–cm, whereas the pattern for amphidinolide H is m–cm–c. While they share a common carbon framework, these two macrolides are not from the same strain of *Amphidinium* (Fig. 5) (Sato *et al.*, 2000; Tsuda *et al.*, 2001).

B. Amphidinols

A stable isotope incorporation study has been reported for amphidinols 2, 3, and 4 (Fig. 5) (Houdai *et al.*, 2001). Amphidinols 3 and 4 showed identical patterns of carbon incorporation. These polyhydroxy

polyenes appear to be more polyketide-like in their construction than the amphidinolides having as many as nine contiguous acetate units. Also present are three c–m–m and one c–m–m(m). Unlike the amphidinolides, acetate was not incorporated at all positions. Carbons 1 and 2 remained unlabeled by either [1-^{13}C], [2-^{13}C]. This and the presence of vicinal hydroxyls at C1 and C2 suggest that the starter unit for this polyketide may be glycolic acid, which is also the starter unit for OA and the ester portion of DTX-4 (Needham *et al.*, 1994, 1995). Although amphidinols 2 and 4 share an identical carbon backbone, their acetate incorporation patterns were not the same. The first C1 deletion occurs between carbons 11 and 12 in amphidinol 2 and between carbons 21 and 22 in amphidinol 4. Thus the labeling patterns are reversed between C12 and C21 and restored at C22. AM2 has methyl substituents, derived from C2 of acetate at C17 and C19.

C. Dinophysis Toxins

Stable isotope incorporation experiments have been performed on DTX-5a and -5b (Fig. 5) (Macpherson *et al.*, 2003). Incorporation patterns of the OA portion of these two analogs were revealed to be identical to DTX-4 (Needham *et al.*, 1995; Wright *et al.*, 1996). DTX-5a and -5b have very similar side chains, the only difference being the number of carbons in the diol portion of the ester side chain. This portion of DTX-5b is identical to DTX-4. However, DTX-5a has one fewer carbon in this region. Earlier experiments with DTX-4 indicated that this polyketide was constructed of contiguous acetate units and no C1 deletions were observed (Needham *et al.*, 1995; Wright *et al.*, 1996). Further, the ester portion of this side chain was introduced by the Baeyer-Villiger oxidation of a continuous polyketide chain. One bond coupling between carbons at 8′ and 1″ of DTX-4 revealed that these two carbons originated from the same acetate unit. A similar pattern was observed for this region of DTX-5b. This was not the case for DTX-5a. Whereas the ester carbonyl (C1″) of DTX-4 and -5b is derived from C1 of acetate, in DTX-5a it is derived from C2 of acetate. Further, labeling studies revealed that this carbon was not coupled to the adjacent carbon suggesting a deletion of C1 of acetate. The amide containing regions of DTX-5a and -5b are identical, with the Baeyer-Villiger oxidation occurring exactly 14 carbons and 1 nitrogen from the sulfated end in both. Two important conclusions were drawn from this observation. First that the Baeyer-Villiger oxidation is highly regioselective and must have occurred after the C1 deletion in order to place the ester exactly the same distance from the sulfated end of both analogs.

Second, the Favorskii-like deletion of C1 of acetate must be an integral part of the polyketide chain assembly process. Another interesting observation is the incorporation of a single glycine at carbons 8″ and 9″ and at the nitrogen of DTX-5a and -5b. This suggests that the side chain of DTX-5a and -5b is constructed from a mixed polyketide synthase/nonribosomal peptide synthetase type enzyme. This is the first and only demonstration of the incorporation of an amino acid into a dinoflagellate derived polyketide.

D. Yessotoxin

Finally, stable isotope incorporation studies were reported for yessotoxin (Fig. 5) (Satake, 2000). Like all other dinoflagellate-derived polyketides, extensive deletions of C1 of acetate were observed. Yessotoxin is composed of three intact c–m units, eight c–m–m, three c–m–m–(m) m, and one c–m–m(m)–m(m) and one isolated m. Six pendent methyl groups and one methylene are present in YTX with only one at C19 derived from S-adenosyl methionine.

IV. Polyketide Biosynthesis at the Molecular Level

The identification of the genes for dinoflagellate polyketide biosynthesis is an attractive goal for a variety of reasons. However, there have been no reports of a characterized secondary metabolic pathway from a dinoflagellate. Furthermore, results of this nature may be slow in coming because of the obstacles presented by this group of organisms. This can include the ability (or inability) to culture the organism of interest. For example, *Dinophysis* species have not been maintained in long-term culture. For planktonic dinoflagellates adequate culturing is invariably in liquid medium. To date, the transition to solid media has not been overcome for most species. This seemingly straightforward step has a significant impact on classical methods of gene isolation. Typically, chemical or UV treatment is employed to create mutants. Since dinoflagellates usually have large genomes, 10^4–10^7 mutants would have to be generated to have a high-statistical probability of creating a single cell with a *pks*$^-$ genotype (Plumley, 1997). The mutagenized cells are subsequently plated on agar-solidified medium, although this step could theoretically be carried out in liquid medium. Regardless, the most difficult obstacle is distinguishing desired mutagenized cells from wild type cells. In primary metabolic studies, the surviving mutants typically display a distinctive phenotype (e.g., growth on a selection medium). In the case of secondary metabolite production (i.e.,

polyketide biosynthesis), the identification of a mutant phenotype would require the screening of individual mutants by a single polyketide metabolite assay (e.g., HPLC or ELISA) or single *PKS* gene assay (e.g., PCR).

The most closely related organism to dinoflagellates proven to have a Type I PKS is *Cryptosporidium parvum*, an apicomplexan. Initially, a Type I fatty acid synthase (FAS) (CpFAS1; Genbank Accession AAC99407) was found in *C. parvum* by screening a *HindIII* and *EcoRI* genomic DNA library with a FAS amplicon from *C. parvum* (Zhu *et al.*, 2000). The CpFAS1 ORF spans 25 kb and contains a starter module, three complete modules, and a terminal reductase. The starter module contains a loading domain, which has sequence similarity to ATP-dependent CoA ligases, and an acyl carrier protein (ACP). Each of the three modules has a full set of enzymatic domains (i.e., AT, KS, KR, DH, ER, and ACP) with the expected, conserved functional motifs. Surprisingly, the terminal domain is not a TE, but instead it has high-sequence similarity to a yeast α-aminoadipate reductase. This suggests *C. parvum* releases the tethered product by a nonhydrolytic mechanism like yeasts and fungi (Schweizer *et al.*, 1986). The *CpFAS1* gene architecture resembles an FAS because the full set of domains are present in each module. However, the presence of multiple modules is suggestive of a PKS and the putative CpFAS1 is actually more similar to PKSs in a phylogenetic analysis (Snyder *et al.*, 2005). A genome sequencing survey project of *C. parvum* made possible the discovery of the first Type I PKS (CpPKS1) in any protist (Zhu *et al.*, 2002). The *CpPKS1* gene spans an intronless 40 kb and contains seven PKS modules. The loading module and terminal reductase are similar to those found in *CpFAS1*. Only two AT domains are found within the entire gene leading to three possibilities: (1) the use of an AT acting *in trans*, (2) the two CpPKS1 AT domains have multiple roles, or (3) the KS domains in the AT-lacking modules accept the acyl moieties directly. A portion of CpPKS1 was created synthetically, and polyclonal chicken antibodies were raised against the peptide. The anti-CpPKS1 antibodies localized presumably to the native CpPKS1 protein in *C. parvum* sporozites. This was observed by immunofluorescence microscopy.

Despite the obstacles that still need to be overcome when dealing with dinoflagellates, advances have been made in the identification of putative polyketide synthase genes from marine dinoflagellates (Snyder *et al.*, 2003). Degenerate primers for the ketosynthase domain of Type I PKSs and Type II PKSs were tested against nine strains (representing six genera and seven species) of dinoflagellates by PCR and RT-PCR. Seven of the nine strains yielded products that were

homologous with known and putative Type I PKSs. In each case, the presence of a PKS gene was correlated with the presence of bacteria in the cultures as identified by amplification of the bacterial 16S rRNA gene. Clearly, the origin of the amplified PKSs remained debatable, and this was addressed from several angles. Southern hybridization was used to demonstrate the presence of highly methylated DNA extracted from dinoflagellate cultures by the ineffectiveness of methylation-sensitive restriction enzymes. Furthermore, some of the PKS amplicons hybridized to the highly methylated (i.e., dinoflagellate) DNA for some species. Overall, an amino acid phylogenetic comparison of the PKS amplicons showed a general dispersion rather than a grouping with clades of bacterial or fungal Type I PKSs. Likewise, the PKSs amplified from dinoflagellate cultures did not form a clade either, with the exception of three PKSs from *K. brevis* (Wilson). These three *K. brevis* PKSs also grouped with the CpFAS1 ketosynthases, and recall the CpFAS1 is *very* similar to PKSs in the National Center for Biotechnology Information (NCBI) database. However, *K. brevis* has not been grown axenically. The associated bacteria might be the source of the toxins or the PKS genes.

A more detailed analysis was undertaken to confirm the origin of the three *C. parvum*-like PKSs from *K. brevis*. A PCR survey using sequence-specific primers against five strains (six isolates) of *K. brevis* revealed all three of these PKSs in all of the isolates. Furthermore, these PKS-encoding genes were localized to *K. brevis* by a combination of flow cytometry/PCR and FISH. A *K. brevis* culture was subjected to flow-cytometric cell sorting based on size and chlorophyll autofluorescence. Dinoflagellate cells were successfully sorted from bacterial cells; however, bacterial cells were not successfully separated from dinoflagellate cells. This was confirmed by the presence (or absence) of 18S and 16S rRNA genes in a PCR assay. Sequence-specific PCR (same as done in the *K. brevis* survey) indicated all three PKS genes were present in the dinoflagellate fraction, but only one of these three was also present in the bacterial fraction. Whole-cell FISH confirmed the presence of the same two PKSs not found in the bacterial fraction by labeling to the *K. brevis* cells and not to associated bacteria. The third PKS found in the bacterial fraction labeled a bacteria-sized coccoid-like particle and *K. brevis* to a lesser extent. Thus, two genes localized exclusively to *K. brevis* cells while a third localized to both *K. brevis* and associated bacteria. While these genes have not yet been linked to toxin production, the work described the first definitive evidence of resident PKS genes in any dinoflagellate.

Polyether ladders, such as the brevetoxins and yessotoxin, are entirely unique to dinoflagellates. There is, therefore, no precedent to look to for clues as to the processes that would be required for their biosynthesis. The closest analogy would probably be the polyether metabolite monensin, which is biosynthesized by the soil-borne *Streptomyces cinnamonensis*. This nonladder polyether provided the first example of a sequenced Type I PKS polyether gene cluster (Leadlay *et al.*, 2001). The only other nonladder polyether with a sequenced Type I PKS gene cluster is nanchangmycin (Sun *et al.*, 2003). The biosynthetic process for both of these polyethers includes ketoreductases and dehydratases for the formation of the backbone carbon–carbon double bonds. The alkenes are proposed to be epoxidized and undergo a polyepoxide cyclization to form the ether linkages (Fig. 6). The identification of a putative flavin-linked epoxidase and epoxide hydrolases (for cyclization) in these gene clusters support this hypothesis. In the case of monensin, deletions of these genes abolished production of the polyether (Oliynyk *et al.*, 2003). The carbon backbone of brevetoxin is predicted to be a mostly all *trans* polyene that is epoxidized and

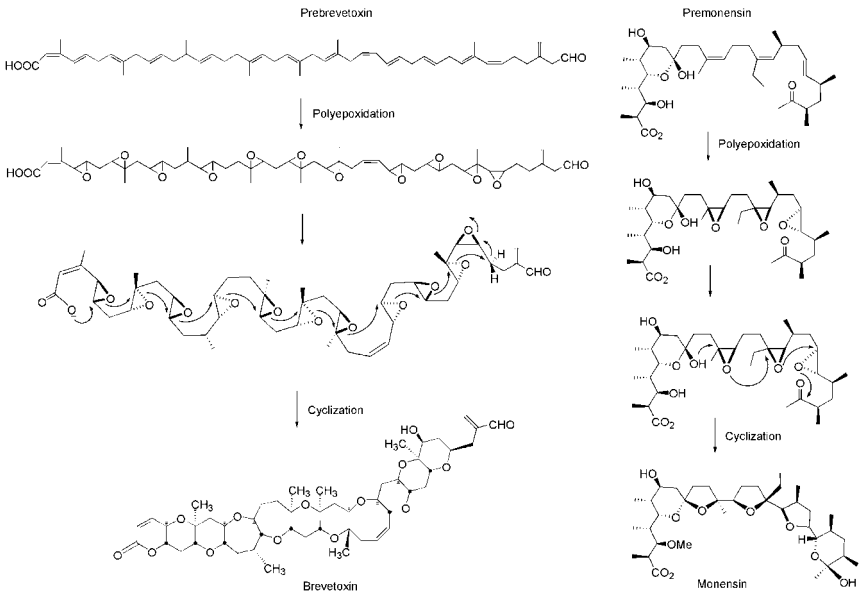

FIG. 6. Proposed mechanism for brevetoxin production and monensin biosynthesis. Both polyenes are epoxidized and undergo cyclization.

undergoes a similar polyepoxide cyclization (Fig. 6) (Lee *et al.*, 1989). Evidence supporting this hypothesis in another dinoflagellate polyketide has been shown by ^{18}O incorporation from molecular oxygen into rings D and E of dinophysistoxins, suggesting a β-epoxidation intermediate (Murata *et al.*, 1998).

V. Current Methods for Isolating Dinoflagellate Genes

Since most dinoflagellate genomes are large, the discovery of novel genes has mostly been achieved through partial sequencing of cDNA libraries, (i.e., ESTs). This approach identifies thousands of genes and is not hindered by genome size or introns. Recently, about 7000 ESTs were sequenced yielding 5280 unique gene clusters from *K. brevis* (Wilson) (Lidie *et al.*, 2005). Only 1556 (29%) of these genes showed similarity to previously identified genes using a cutoff of $P < 10^{-4}$ in the BLASTx search algorithm. The genes that met this criteria were involved in metabolism (23%), signal transduction (20%), transcription/translation (15%), and structure/cytoskeleton (11%). This library was submitted to two databases, Genbank at NCBI (www.ncbi.nlm.nih.gov) and Marine Genomics (www.marinegenomics.org). In the latter, the sequences can be searched based on gene ontology. Searches for PKS-related genes are shown in Table III. Search terms were polyketide, ketosynthase, transacylase, acyl-carrier protein, and thioesterase. Other PKS-related search queries that did not return any sequences were acyltransferase (yet transacylase did), ketoreductase, enoylreductase, and dehydratase. Curiously the PKS genes found previously were not identified in the EST database.

One of the greater purposes for the creation of the *K. brevis* EST library is the development of a DNA microarray (Lidie *et al.*, 2005). When optimized and normalized, this tool enables genomewide investigations into the mechanisms that regulate the growth and toxicity of *K. brevis* by comparing expression levels under different environmental conditions. Clearly, an EST library is an effective and informative method for the acquisition of new sequences; however, it requires a substantial amount of resources. The remainder of this chapter focuses on techniques used to identify specific genes.

Despite being part of the enormous *Gonyaulax polyedra* (=*Lingulodinium polyedrum*) genome, the full-length luciferase mRNA was one of the first targeted genes to be characterized from any dinoflagellate (Bae and Hastings, 1994). Two regions of the mRNA were found using an antibody versus luciferase and a cDNA expression library, and the full mRNA was found by Northern hybridization. Later, the

structure and organization of the *luciferase* gene was characterized by a combination of Southern analysis, inverse PCR, the 5′ rapid amplification of cDNA ends (RACE), and an RNase protection assay (Li and Hastings, 1998).

Functional assignments for novel genes can only be made indirectly when they are identified by degenerate-oligonucleotide–primed PCR or mass sequencing of genomes or cDNAs. Heterologous functional complementation of auxotrophic yeast allows both the isolation and functional confirmation of genes. Essentially, a prototrophic phenotype in an auxotrophic organism is restored by transformation with a gene or gene library derived from another organism. This technique has been used in the case of *Crypthecodinium cohnii* (Lippmeier *et al.*, 2002). The study used a mutant *Saccharomyces cerevisiae* deficient in its ability to biosynthesize adenine. The auxotrophic *S. cerevisiae* was transformed with a *C. cohnii* cDNA library that was cloned into a yeast expression vector, pFL-61. Of 90,000 primary transformants, only one was able to cure adenine auxotrophy. Specifically, the *ade2* phenotype was restored by a 2468 bp complementing cDNA (AY032657). Complementation of phenotypes showing deficiencies in leucine and tryptophan biosynthesis were also attempted but not observed.

Functional complementation was also used to isolate and confirm the function of a cyclin from *G. polyhedra* (Bertomeu and Morse, 2004). Cyclins control the timing and location of activated cyclin-dependent kinases (CDKs), and CDK activity is necessary for the cell's entry into the M and S phases of mitosis. The mechanics of cell division in dinoflagellates, particularly the harmful species, is important for understanding cell proliferation to high levels (i.e., blooms). In addition, the cell cycle in *Gonyaulax* is linked to the circadian clock. The experimental design used yeast, harboring mutations in either G1- or M-phase cyclins. They also had a distinct Gal1 promoter inducible cyclin. Thus, media containing galactose was necessary for wild-type survival in the absence of complementation. The transformation was performed with a *G. polyhedra* cDNA library that was cloned into a modified pYES2 yeast expression vector. The selectable marker for this vector is *URA3*, which confers the ability to synthesize uracil. Thus, transformants were grown in the absence of uracil, which allowed for successful transformation selection. Also, galactose was not present in the media, which prevented the growth of nontransformants.

Essentially, two unique sequences were identified from the 136 transformants. One of the sequences, GpCyc1, showed a low similarity (∼30%) to cyclins from other organisms, which is typical for the similarity of cyclins from different groups. However, GpCyc1 showed a

TABLE III

PKS-Related ESTs from *K. brevis* and Their Translated BLAST Best Match in the GENBANK nr Database*

ESTMGID #	EST accession #	Translated BLAST best match	% identity/% positive	Score/e value	Proposed function (active site)
4	CO059032	Malonyl CoA-ACP transacylase [*Arabidopsis thaliana*]	39/56	112/1e-23	Acyltransferase
2796	CO063201	Acetyl-CoA acyltransferase, [*Bos taurus*]	70/83	186/7e-46	Acyltransferase
1008	CO517375	Type I polyketide synthase [*Mycobacterium avium*]	35/53	108/2e-22	Ketosynthase (DTECCSA)
4332	CO064722	Type I polyketide synthase [*Nostoc punctiforme*]	37/54	71.6/8e-12	Ketosynthase (DTACSAS)
5361	CO059138	Type I polyketide synthase [*Anabaena variabilis*]	37/55	135/1e-30	Ketosynthase (DTACSAS)
6736	CO060493	Type I polyketide synthase [*Nostoc* sp. *PCC 7120*]	42/57	157/5e-37	Ketosynthase (DTACSAS)
4718	CO065100	Type I polyketide synthase [*Desulfovibrio desulfuricans*]	33/51	58.9/1e-07	Ketosynthase (N-C termini boundary)
6371	CO060132	Type I polyketide synthase [*Streptomyces rochei*]	30/47	63.5/7e-09	Ketosynthase (N-C termini boundary)
4674	CO065056	Acyl-CoA thioesterase [*Strongylocentrotus purpuratus*]	48/67	108/7e-23	Thioesterase
6862	CO060619	Acyl-CoA thioesterase [*Strongylocentrotus purpuratus*]	48/67	107/2e-22	Thioesterase

*The listed EST accession numbers correspond to the ESTs MGID (marine genomics identification number).

high similarity (77%) to an EST sequence from *Alexandrium*. This line of support along with GpCyc1 having a typical *Gonyaulax* GC content (66.5%) and codon usage suggests it originated from the dinoflagellate. While this method is effective for yeast lacking a critical gene for primary metabolism or cell division, its application to polyketide biosynthesis (i.e., secondary metabolism) may be limited.

Differential display (DD) is a molecular tool used to analyze differences between complex genomes at the level of gene expression. This allows the analysis of differentially expressed genes in eukaryotes. Two or more sets of differentially expressed mRNAs to be compared are used as templates to generate the corresponding cDNAs. The mRNAs are reverse transcribed using a degenerate oligo (dT) primer with the general sequence 5′-T_nVN ($n = 11$–12). Thus, there are 12 permutations of the last two 3′ bases, and any particular primer (e.g., 5′-T_{11}CA) will recognize {1/12} of the total mRNA population. A portion of the cDNAs is then amplified in a PCR reaction containing one random decamer primer, the respective modified oligo (dT) primer, and [α-P^{32}]dATP. Following the amplification, the products are separated by polyacrylamide gel electrophoresis, and the bands are visualized by autoradiography. Bands of desired size are excised and reamplified with the same pair of primers. Finally, the products are sequenced. Usually, the purpose is to identify bands unique to one physiological state (i.e., genes correlated with growth phase or environmental stresses). This could also be applied to two strains of the same organism with the goal of identifying genes contributing to a characteristic of one of the two strains.

In this regard, DD has been applied to three toxic and three nontoxic strains of *Alexandrium tamarense* (Taroncher-Oldenburg and Anderson, 2000). However, the inter- and intrageneric variabilities were high. Thus, there were no shared expressed genes in all three toxic strains that were also not present in the nontoxic strains. More success was achieved using synchronized cultures of *A. fundyense*. In this case, the DD patterns were identical for G_1, S, and G_2 phases, which permitted the identification of differentially expressed bands. Saxitoxin has been shown to accumulate during a discrete time period in the G_1 phase of the cell cycle (Taroncher-Oldenburg *et al.*, 1997). Thus, the focus was to identify genes expressed or suppressed in early G_1 when compared with the remainder of the cell cycle. Three genes met the criteria, and these coded for S-adenosylhomocysteine hydrolase, methionine aminopeptidase, and a histone-like protein. Both S-adenosylhomocysteine hydrolase (in SAM regulation) and methionine aminopeptidase (in protein synthesis processing) theoretical-

ly could play a secondary role in saxitoxin biosynthesis (e.g., regulation), but they probably do not contribute directly.

VI. Conclusions

Stable isotope feeding studies early on in dinoflagellate biochemical investigations firmly established a polyketide origin for many metabolites. The next step will be linking a gene to toxin production in a dinoflagellate. The advances in molecular biology will help to identify gene clusters from these organisms, establish the role of these genes and understand how they are regulated under varying environmental conditions. Growing use of techniques like flow-cytometry, functional complementation, and differential display will contribute to characterizing novel dinoflagellate genes. However, the genomewide approaches will lead to the identification of gene clusters. Eventually there will come a time when genome sequencing will become practical for these complex microbes. Presently, there is growing support for an effort to sequence a *Symbiodinium* genome. In combination with genomic data, DNA microarrays will help to understand the role the environment plays on the expression of polyketide biosynthetic genes and hopefully how it can be influenced to protect and enhance human health. The demand for novel polyketides in uses like medicinals, as molecular tools and in understanding toxin biosynthesis will continue to drive research in this field.

Acknowledgments

This work was supported in part by NIH/NIEHS S11 ES11181.

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Biological Halogenation has Moved far Beyond Haloperoxidases

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I. Introduction

In 1961, Petty called halogenated metabolites ''chance products of nature.'' This was due to the fact that until 1961 only 29 halogenated organohalogen compounds had been isolated from living organisms. The first metabolite whose structural analysis showed that it contained a halogen atom was found in the marine eukaryote *Gorgonia cavolinii*, in 1896 (Drechsel, 1896). This compound, 3,5-diiodotyrosine (Fig. 1), was later also isolated from the thyroid glands of mammals (Foster, 1929).

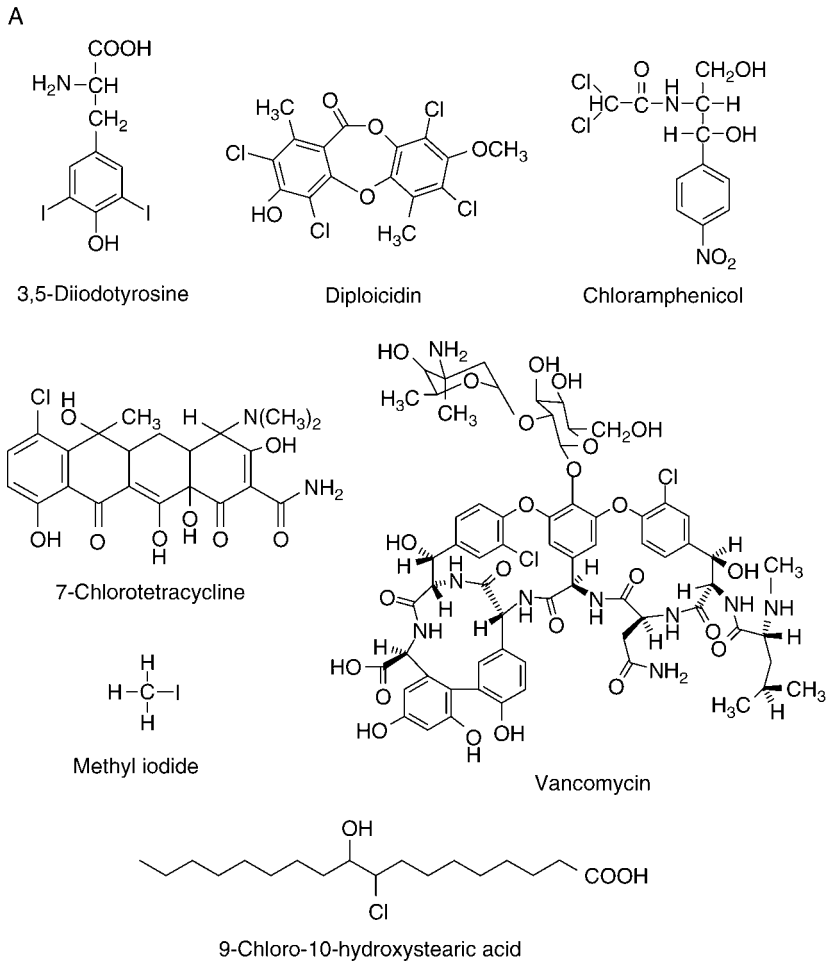
The first halogenated metabolite identified in a microorganism was diploicidin (Zopf, 1904) (Fig. 1). During the intensive search for antibiotics after the detection of penicillin, halogenated antibiotics, such as chloramphenicol (Ehrlich *et al.*, 1947) (Fig. 1), 7-chlorotetracycline (Duggar, 1948) (Fig. 1), vancomycin (McCormick *et al.*, 1956) (Fig. 1),

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 59 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(06)59005-7

and many others were isolated. Until now, more than 4000 organohalogens are known to be produced by living organisms (Gribble, 2004).

There is an enormous structural variety of halogenated metabolites, starting from structurally simple compounds, such as methyl iodide, methyl bromide, and methyl chloride (Cowan *et al.*, 1973; Harper, 1985) (Fig. 1), halogenated fatty acids (Schmitz and Gopichand, 1978; White and Hager, 1977) (Fig. 1), to aliphatic amino acids (Sanada *et al.*, 1986) (Fig. 1), nonaromatic cyclic compounds (Clutterbuck *et al.*, 1940) (Fig. 1), nonaromatic heterocycles (Takahashi *et al.*, 1999) (Fig. 1),



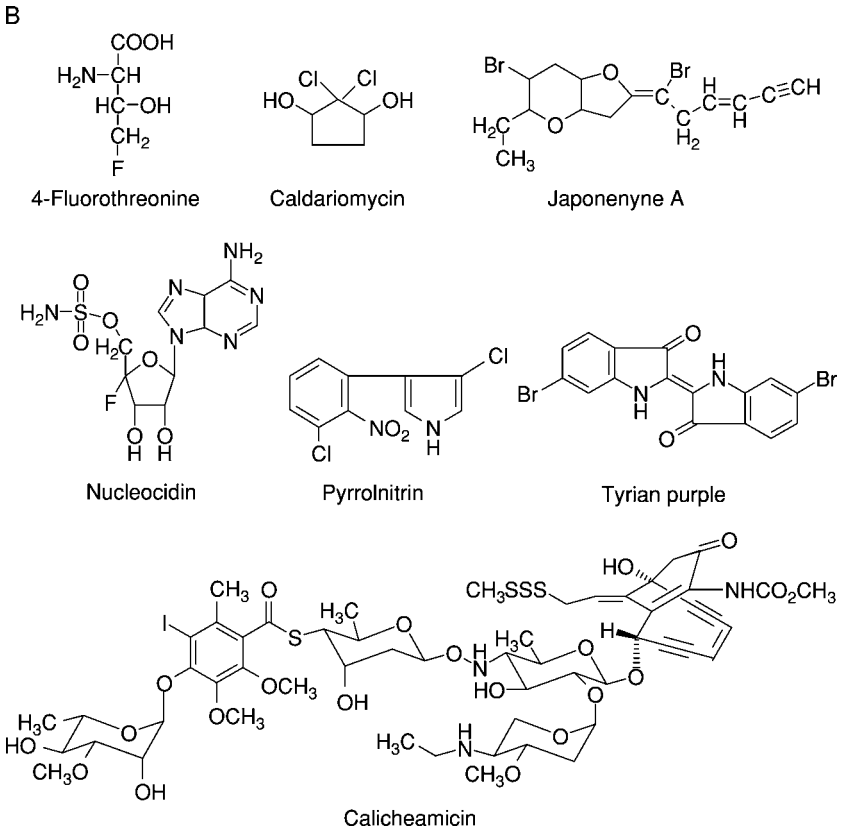


FIG. 1. Chemical structures of halometabolites showing their structural diversity.

carbohydrates (Morton *et al.*, 1969) (Fig. 1), phenolic compounds (Drechsel, 1896; Foster, 1929) (Fig. 1), pyrrole (Arima *et al.*, 1964) and indole derivatives (Friedländer, 1909) (Fig. 1), and structurally complicated metabolites, such as enediynes (Lee *et al.*, 1987) (Fig. 1), aromatic polyketides (Duggar, 1948) (Fig. 1), and glycopeptide antibiotics (McCormick, 1956) (Fig. 1). Although the structural diversity is enormous, it is noteworthy that in the case of the phenolic compounds the position of the halogen atoms is either in *o*- or/and *p*-position to a hydroxyl group, but never in the *m*-position, and there are no halometabolites containing a halogen atom at a nonhydroxylated phenyl ring.

The number of producing organisms is comparable to the huge structural diversity of halometabolites. Organohalogen compounds have been isolated from bacteria, fungi, algae, lichen, higher plants,

invertebrates, and vertebrates (Gribble, 1996). However, so far halometabolites have not been detected in anaerobic organisms.

There are some general features concerning the production of organohalogen compounds by living organism. Terrestrial organisms normally synthesize chlorinated compounds; however, when chloride is substituted by bromide in the culture medium, the corresponding brominated metabolites can be obtained (Ajisaka *et al.*, 1969; Bister *et al.*, 2003; Doerschuk *et al.*, 1959; Ezaki *et al.*, 1983; Sánchez *et al.*, 2005; Smith, 1953). Brominated organic compounds are the most abundant halometabolites, and they are predominantly produced by marine organisms. Compared to organochlorine and organobromine compounds, only very few iodinated metabolites have been isolated (Gribble, 1996). In contrast to the bromoanalogues of chlorinated biological products, the iodoanalogs cannot be obtained by substitution of chloride ions by iodide ions in the culture medium. The number of fluorinated metabolites is also very low, although fluorine is the 13th most abundant element in the earth's crust. However, the concentration of fluoride in surface water is low and the fluoride ion is tightly hydrated in water and effectively inert (O'Hagan and Harper, 1999). Organofluorine compounds have only been isolated from bacteria and higher plants (Marais, 1943; Sanada *et al.*, 1986).

The functions of these organohalogen compounds in and for the producing organism are only known for a few halometabolites. The biological function of thyroxine as a thyroid hormone in mammals is well known. Some plants produce 4-chloroindole-3-acetic acids that act as potent plant growth hormone (Böttger *et al.*, 1978) and in *Dictyostelium discoideum* (3,5-dichloro-2,4,6-trihydroxyphenyl)-1-hexane-1-one functions as a signal molecule that triggers the transformation of undifferentiated cells into fruiting bodies (Morris *et al.*, 1987). A number of organohalogen compounds produced by marine algae and some terrestrial plants have antifeedant activity (Paul, 1987). Although many halometabolites show antibiotic or antitumor activity, their biological function for the producing organism is not known.

II. Haloperoxidases and Perhydrolases

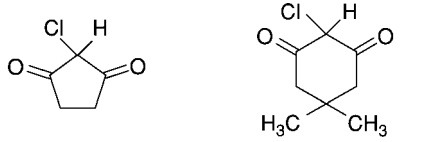
A. Haloperoxidases

1. *Heme Haloperoxidases*

Caldariomycin is an antibiotic produced by the mold *Caldariomyces fumago*. According to biosynthetic studies, caldariomycin is derived from shikimic acid (Beckwith *et al.*, 1963) and β-ketoadipic acid (Shaw

et al., 1959) was suggested to be an intermediate in the formation of the 1,3-cyclopentanedione, a late intermediate in caldariomycin biosynthesis which would then be chlorinated and reduced to the final product caldariomycin (Fig. 1) (Beckwith and Hager, 1963). With β-ketoadipic acid as the substrate, Shaw and Hager (1959) demonstrated chlorinating activity leading to the formation of δ-chlorolevulinic acid. However, the very low incorporation of δ-chlorolevulinic acid into caldariomycin suggested that δ-chlorolevulinic acid is no real intermediate in caldariomycin biosynthesis. Beckwith and Hager (1963) showed that 1,3-cyclopentanedione and 2-chloro-1,3-cyclopentanedione were also substrates for the chlorinating enzyme. The final product of this enzymatic chlorination reaction, 2,2-dichloro-1,3-cyclopentanedione, was converted to caldariomycin by *Caldariomyces fumago*.

The chlorinating enzymes from *Caldariomyces fumago* was found to be an extracellular enzyme and shown to require hydrogen peroxide, chloride ion, and a suitable organic substrate for chlorinating activity and was thus named chloroperoxidase. Detailed studies of the enzyme revealed that it contains ferriprotoporphyrin IX as the prosthetic group (Morris and Hager, 1966). In early studies, radioactive chloride was used to monitor chloroperoxidase activity. Based on the structural similarity between 2-chloro-1,3-cyclopentanedione and monochlorodimedone (Fig. 2), Hager *et al.* (1966) developed a spectrophotometric assay for haloperoxidases which allowed the continuous monitoring of chlorinating and brominating activity. In this context it has to be noted that monochlorodimedone is a purely synthetic compound; it has never been isolated from a biological source. The monochlorodimedone assay was subsequently used by every group working on halogenating enzymes, and many heme-containing haloperoxidases (bromo- and chloroperoxidases) have thus been detected from a variety of different



2-Chloro-1,3-cyclopentanedione

Monochlorodimedone

FIG. 2. Chemical structure of 2-chloro-1,3-cyclopentanedione, an intermediate in caldariomycin biosynthesis and the synthetic compound monochlorodimedone, which was used in the search for haloperoxidases.

organisms such as marine algae, sea urchin, mammals, and bacteria (Neidleman and Geigert, 1986).

To be able to detect haloperoxidases using the monochlorodimedone assay, these enzymes must have a broad substrate specificity. In fact, it turned out that haloperoxidases accept organic compounds, which are susceptible to electrophilic attack and the regioselectivity was not different from that seen in electrophilic chemical halogenation reactions. The reason for this lack of substrate specificity and regioselectivity became evident when the three-dimensional structure and the reaction mechanism of chloroperoxidase from *Caldariomyces fumago* were elucidated (Sundaramoorthy *et al.*, 1998). Haloperoxidases oxidize chloride and bromide using hydrogen peroxide as the oxidant. The thus formed hypochlorous and hypobromous acids leave the active site of the enzyme and attack organic compounds activated for an electrophilic substitution in a nonenzymatic reaction (Fig. 3A).

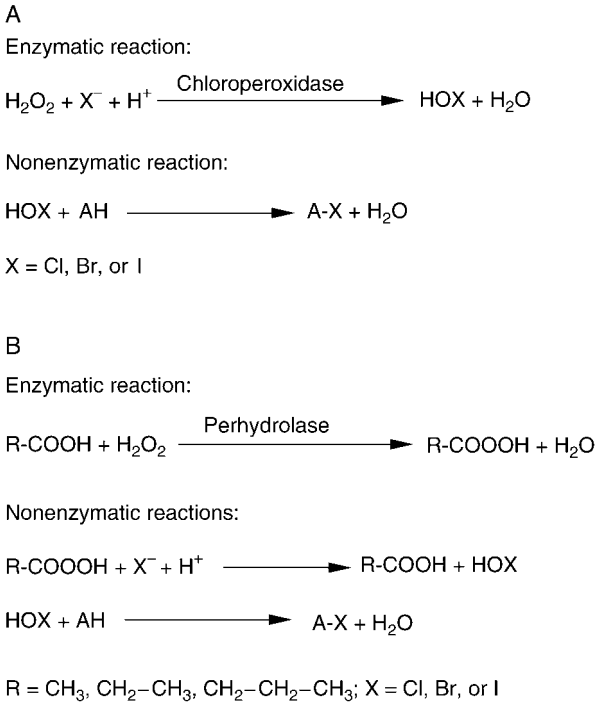


FIG. 3. Enzyme-catalyzed reactions and subsequent nonenzymatic halogenation reactions of (A) heme- and vanadium-containing haloperoxidases and (B) perhydrolases.

Since the actual halogenation reaction is not catalyzed by the enzyme, halogenation reactions initiated by haloperoxidases show the same regioselectivity as electrophilic chemical halogenation. Together with the fact that although haloperoxidases have been isolated from organisms known to produce organohalogen compounds, it has never been demonstrated that these haloperoxidases are actually involved in the biosynthesis of these halometabolites, this raises the question whether haloperoxidases are actually the type of halogenating enzymes involved in the biosynthesis of secondary metabolites in microorganisms.

2. *Vanadium Haloperoxidases*

In 1984, Vilter reported the detection of a novel haloperoxidase, which did not contain heme but instead required vanadium for halogenating activity. Originally, this enzyme from the brown alga *Ascophyllum nodosum* was identified as an iodoperoxidase, but using the monochlorodimedone assay it could be shown that it was actually a bromoperoxidase (Vilter, 1983). Subsequently, many other vanadium-containing chloro- and bromoperoxidases were isolated from other marine algae, lichen, and fungi, but not from bacteria. Detailed characterization of the enzymes (De Boer and Wever, 1988; van Schijndel *et al.*, 1994) and elucidation of the three-dimensional structure of the chloroperoxidase from the fungus C*urvularia inaequalis* (Messerschmidt and Wever, 1996) showed that these enzymes, like the heme-containing haloperoxidases, produce hypohalous acids which then react in a nonenzymatic reaction with suitable organic compounds (Fig. 3A). Thus, vanadium-containing haloperoxidases also lack substrate specificity and regioselectivity, which makes it very unlikely that they are involved in the biosynthesis of secondary metabolites. So far, no vanadium-containing haloperoxidase could be linked to the biosynthesis of a halometabolite in any organism.

B. Perhydrolases

Whereas heme-containing bromoperoxidases have been isolated from bacteria, vanadium haloperoxidases have not been detected in bacteria, so far. However, using the monochlorodimedone assay, Wiesner *et al.* (1988) described the isolation of a nonheme chloroperoxidase from the pyrrolnitrin producer *Pseudomonas pyrrocinia*. This chloroperoxidase did neither contain any metal ions nor any other cofactor. Based on the requirement of hydrogen peroxide for halogenating activity, the enzyme was considered to be a haloperoxidase. Similar enzymes were also isolated from a number of other

antibiotic producing bacteria (van Pée, 1996). Comparison of the amino acid sequences and the elucidation of the three-dimensional structure of the enzyme from the 7-chlorotetracycline producer *Streptomyces aureofaciens* Tü24 revealed that these enzymes have an α/β hydrolase fold and show a high degree of similarity to esterases (Hecht *et al.*, 1994). Structural investigations and comparison of several of these metal-free, cofactor-independent halogenating enzymes showed that they act as perhydrolases in the presence of short-chain fatty acids and hydrogen peroxide catalyzing the formation of peracids. These peracids then react in an enzyme-independent reaction with halide ions leading to the formation of hypohalous acids as the actual halogenating agent (Fig. 3B). This mechanism is consistent with the lack of substrate specificity and regioselectivity also found for the halogenation reactions initiated by these enzymes. Thus it was clear that these enzymes were not peroxidases, but belonged to the serine hydrolase family, instead. The question, why some members of this family show perhydrolase activity whereas others do not, was answered by Bernhardt *et al.* (2005). Comparison of the active site structure of an esterase from *Pseudomonas fluorescens* and a Leu29Pro mutant with increased perhydrolase activity and concomitantly reduced esterase activity revealed that the increase in perhydrolase activity was due to an additional hydrogen bond between a carbonyl group in the vicinity of the active site that serves as a means to stabilize hydrogen peroxide attack on the acyl-enzyme intermediate (Fig. 4).

Since perhydrolases as well as haloperoxidases produce free hypohalous acids in the presence of bromide and chloride, they are unlikely to be involved in halometabolite biosynthesis. That perhydrolases are not involved in the synthesis of halometabolites was proven for the biosynthesis of the antifungal antibiotic pyrrolnitrin (Fig. 5). The inactivation

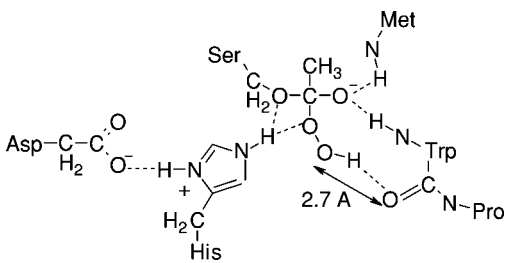


FIG. 4. Proposal for the molecular basis for perhydrolase activity in esterases. An additional hydrogen bond in the leucine29proline mutant facilitates and subsequently stabilizes the formation of the second tetrahedral intermediate.

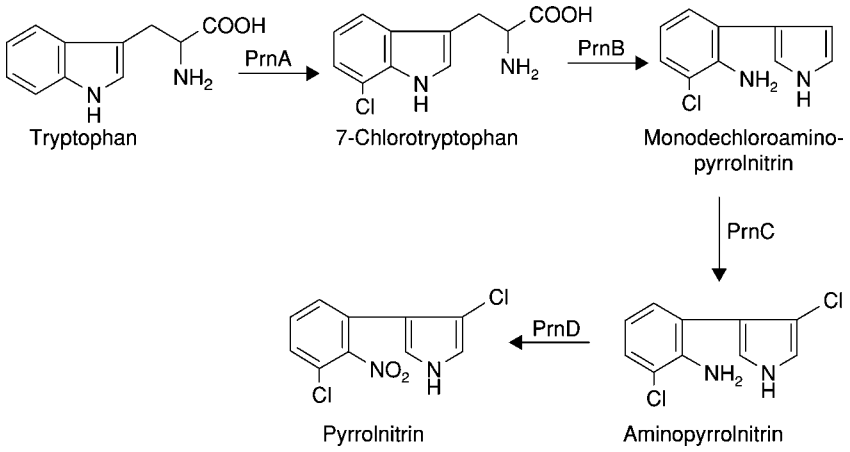


FIG. 5. Pyrrolnitrin biosynthetic pathway.

of a perhydrolase gene in the pyrrolnitrin producer *Pseudomonas fluorescens* BL915 was shown to have no influence on pyrrolnitrin production (Kirner *et al.*, 1996).

III. Flavin-Dependent Halogenases

Since heme-containing haloperoxidases, as well as vanadium haloperoxidases and perhydrolases, seemed extremely unlikely to be involved in the biosyntheses of halometabolites, the question was what kind of halogenating enzymes are involved in halometabolite biosynthesis and how can they be found? One important point was the substrate used for the screening for halogenating enzymes. With monochlorodimedone, which is not a naturally occurring halometabolite, and hydrogen peroxide only haloperoxidases and perhydrolases had been detected. Since the development of the monochlorodimedone assay information had accumulated that the halogenating enzymes involved in halometabolite biosyntheses are highly substrate specific and regioselective (van Pée, 1996). However, for the detection of such enzymes, monochlorodimedone was not suitable as a substrate. To detect halogenating enzymes involved in halometabolite biosyntheses, their natural substrates must be used. Unfortunately, this kind of reasoning alone did not solve the problem, because either the natural substrates, which were halogenated during the biosynthesis, were not known or not easily or not available at all.

Using a genetic approach by cloning the biosynthetic gene cluster for 7-chlorotetracycline biosynthesis in *Streptomyces aureofaciens* and complementation of mutants, Dairi *et al.* (1995) identified the gene of the halogenating enzyme from 7-chlorotetracyclin biosynthesis. However, no attempts to show *in vitro* activity and to characterize the enzyme have been reported. The amino acid sequence, deduced from the DNA sequence of the gene, clearly showed that the enzyme belonged to a new type of halogenating enzyme that had no similarity to haloperoxidases and perhyrolases at all. However, the sequence did not give any indication to which already-known enzymes this halogenase could be related.

A. Detection of Flavin-Dependent Halogenases

The antifungal antibiotic pyrrolnitrin contains two chlorine atoms (Arima *et al.*, 1964) (Fig. 5). Pyrrolnitrin is known to be synthesized from the amino acid tryptophan, and 7-chlorotryptophan was suggested to be the first intermediate in pyrrolnitrin biosynthesis. For monodechloroaminopyrrolnitrin, it could be demonstrated that it is a true intermediate in pyrrolnitrin formation and is chlorinated in the 3-position of the pyrrole ring to aminopyrrolnitrin (Fig. 5) (van Pée *et al.*, 1980). Thus pyrrolnitrin biosynthesis seemed to be quite well suited for the search for halogenases with a high substrate specificity, since at least one substrate, monodechloroaminopyrrolnitrin, would be available. The cloning of the pyrrolnitrin biosynthetic gene cluster (Hammer *et al.*, 1997) and the subsequent analysis of the function of the four genes of this cluster proved that tryptophan was also a substrate for a halogenase (Kirner *et al.*, 1998). The cluster contains two genes for halogenases with different substrate specificities. One halogenase catalyzes the regioselective chlorination of tryptophan to 7-chlorotryptophan and was thus named tryptophan 7-halogenase (PrnA), and the other catalyzes the regioselective chlorination of monodechloroaminopyrrolnitrin to aminopyrrolnitrin and was named monodechloraminopyrrolnitrin 3-halogenase (PrnC). These two halogenases cannot substitute each other. Comparison of their amino acid sequences showed no significant overall sequence similarity. However, PrnC showed significant similarity to the halogenase from 7-chlorotetracycline biosynthesis. Detailed analysis of the amino acid sequences of the three halogenases revealed that PrnA and PrnC both contained a motif for a nucleotide binding site (GxGxxG) near the amino terminal end of the enzymes, which was missing from the published sequence of the halogenase from 7-chlorotetracycline

biosynthesis (Dairi *et al.*, 1995), due to a sequencing error (Hammer *et al.*, 1997). The existence of a nucleotide-binding site was consistent with the finding that in crude cell-free extracts, PrnA and PrnC required NADH for chlorinating and brominating activity (Hohaus *et al.*, 1997). During purification of PrnA it was soon realized that not only NADH, but also flavin adenine dinucleotide (FAD) was required for halogenating activity. The reason for this could not be explained until it was realized that a second enzyme is also necessary. This second enzyme is a flavin reductase, which catalyzes the reduction of FAD using NAD(P)H as the coenzyme (Keller *et al.*, 2000). The halogenases are specific for $FADH_2$, which is reflected in the fact that the GxGxxG-motif for the nucleotide-binding site is absolutely conserved in all flavin-dependent halogenases detected so far (van Pée and Zehner, 2003). There does not seem to be any specific interaction between the flavin reductase and the halogenase. The flavin reductase present in crude cell-free extracts of the pyrrolnitrin producer *Pseudomonas fluorescens* BL915 in which the PrnA gene was overexpressed could be substituted by flavin reductases from different bacteria, such as SsuE and Fre from *E. coli*, and NADH oxidase from *Thermus thermophilus* (Keller *et al.*, 2000; Unversucht *et al.*, 2005). Unversucht *et al.* (2005) demonstrated that direct contact between the flavin reductase and the halogenase is not required for activity, although halogenating activity is significantly reduced when the two enzymes are physically separated, due to the fact that most of the enzymatically formed $FADH_2$ is destroyed before reaching the halogenase by the reaction with oxygen leading to the formation of flavin hydroperoxide, which decomposes to FAD and hydrogen peroxide. Oxygen has to be present, because it is also necessary for halogenating activity (Keller *et al.*, 2000). The flavin reductase/FAD system can even be substituted by an organometallic Rh-complex [(pentamethylcyclopentadienyl)rhodium-bipyridine], which catalyzes the reduction of FAD using formate as the electron donor (Unversucht *et al.*, 2005). This also shows that it is only $FADH_2$ and not NADH that is required by the halogenase itself for halogenating activity.

However, it is difficult to accept that it is freely diffusible $FADH_2$ that is used by the halogenase in the cell, since due to the reaction with oxygen, which has to be present; this would be a waste of energy and a very inefficient reaction. When the gene cluster for pyrrolnitrin biosynthesis was isolated, only four genes were necessary to promote pyrrolnitrin biosynthesis in the heterologous host *E. coli* (Hammer *et al.*, 1997), and none of these four genes coded for a flavin reductase. In the biosynthetic gene cluster for pyoluteorin also, no flavin reductase

gene was identified (Nowak-Thompson *et al.*, 1999). Whereas in the gene cluster for rebeccamycin biosynthesis from *Lechevalerieria aerocolonigenes* a flavin reductase gene (*rebF*) was detected (Sánchez *et al.*, 2002). The halogenase, which is involved in rebeccamycin biosynthesis, is also a tryptophan 7-halogenase and shows activity when Fre from *E. coli* (Kling *et al.*, 2005) or RebF from the rebeccamycin producer (Yeh *et al.*, 2005) are used as the flavin reductase in the two-component system. The recent sequencing of the genome of the pyrrolnitrin- and pyoluteorin-producing soil bacterium *Pseudomonas fluorescens* Pf-5, however, revealed that the original annotations of the biosynthetic gene clusters were not quite correct. Both gene clusters are larger and contain additional genes, that is, flavin reductase genes among them (Paulsen *et al.*, 2005). Flavin reductase genes have also been detected in the biosynthetic gene clusters for the antitumor polyenone neocarzilin (Fig. 6) from ''*Streptomyces carzinostaticus*'' (Otsuka *et al.*, 2004). These new findings raise the question, whether the producers of halometabolites use distinct flavin reductases that specificially interact with the halogenases to prevent the waste of energy as well as hydrogen peroxide formation.

B. Distribution of Flavin-Dependent Halogenases and Their Natural Substrates

After the detection of the first gene of a flavin-dependent halogenase in the biosynthetic gene cluster for 7-chlorotetracycline biosynthesis (Dairi *et al.*, 1995) and the demonstration of *in vitro* activity of tryptophan 7-(PrnA) and monodechloroaminopyrrolnitrin 3-halogenase (PrnC) from pyrrolnitrin biosynthesis (Hohaus *et al.*, 1997), similar genes were detected in many gene clusters for halometabolites biosynthesis from different organisms (van Pée and Unversucht, 2003; van Pée and Zehner, 2003). These genes were detected during the cloning of biosynthetic gene clusters for halometabolite biosynthesis or the sequencing of whole genomes, and the corresponding enzymes were annotated as halogenases purely on the basis of their sequence similarities with the halogenases from 7-chlorotetracycline and pyrrolnitrin biosynthesis.

In the first few years after the detection of flavin-dependent halogenases, haloperoxidases and perhydrolases were still regarded as halogenating enzymes that might be involved in halometabolite biosynthesis. In the biosynthetic gene clusters for the glycopeptide antibiotics chloroeremomycin (van Wageningen *et al.*, 1998) and balhimycin (Pelzer *et al.*, 1999), one gene coding for a perhydrolase-like enzyme (ORF18 from chloroeremomycin and Bhp from balhimycin

biosynthesis) and one gene coding for a flavin-dependent halogenase (ORF10 from chloroeremomycin and BhaA from balhimycin biosynthesis) were detected. To elucidate whether both enzymes, the perhydrolase Bhp and the flavin-dependent halogenase BhaA, or only one of them were involved in balhimycin biosynthesis, Puk *et al.* (2002) constructed in-frame deletion mutants in the balhimycin producer *Amycolatopsis balhimycina* DSM5908, previously *Amycolatopsis mediterranei* DSM5908. They could clearly show that only the flavin-dependent halogenase BhaA was responsible for incorporation of both chlorine atoms of balhimycin, whereas the perhydrolase-like enzyme Bhp was found to be involved in the formation of β-hydroxytyrosine, a precursor of balhimycin. The genes for flavin-dependent halogenases were also detected in the biosynthetic gene clusters of the glycopeptide antibiotics complestatin (Chiu *et al.*, 2001) and A47934 (Pootoolal *et al.*, 2002), and in the biosynthetic gene cluster of many structurally different halometabolites such as clorobiocin (Eustaquio *et al.*, 2003), simocyclinone (Trefzer *et al.*, 2002), avilamycin A (Weitnauer *et al.*, 2001), and ansamitocin (Yu *et al.*, 2002). In all these halometabolites, the chlorine atoms are attached to a phenolic ring or a pyrrole ring as in the case of pyoluteorin (Nowak-Thompson *et al.*, 1999) and pyrrolnitrin (Hammer *et al.*, 1997), and the halogenases involved in the chlorination of these halometabolites all show sequence similarities to those from 7-chlorotetracyline and PrnC from pyrrolnitrin biosynthesis. Although tryptophan 7-halogenase (PrnA) from pyrrolnitrin biosynthesis is a flavin-dependent halogenase, it does not show significant sequence similarity to the halogenases catalyzing the incorporation of halogen atoms into phenolic or pyrrolic rings. However, halogenases showing sequence similarity to PrnA have been detected in the rebeccamycin (Sánchez *et al.*, 2002) and pyrroindomycin (Zehner *et al.*, 2005) biosynthetic pathways. These halogenases act on tryptophan as a substrate with the halogenase from rebeccamycin biosynthesis (RebH) catalyzing the regioselective chlorination and bromination of tryptophan in the 7-position (Kling *et al.*, 2005; Yeh *et al.*, 2005) like PrnA, and the halogenase from pyrroindomycin biosynthesis catalyzes the incorporation of chlorine and bromine atoms into the 5-position of tryptophan (Zehner *et al.*, 2005). A tryptophan 6-halogenase was found in the thienodolin producer *Streptomyces albogriseolus* (Kling *et al.*, 2005).

The genes for flavin-dependent halogenases have also been found in biosynthetic gene clusters of halometabolites containing the halogen atoms at an aliphatic side chain such as chloramphenicol (Piraee *et al.*, 2004) and the neocarzilins A-C (Otsuka *et al.*, 2004).

Chloramphenicol contains a dichloracetyl group (Fig. 1), and neocarzilin A contains a trichloromethyl and neocarzilin C, a dichloromethyl group (Fig. 6), and thus show important structural similarity in the halogenated part of the molecule to syringomycin E (Guenzi *et al.*, 1998), barbamide (Fig. 6) (Chang *et al.*, 2002), dysidenin, and dysideathiazole (Fig. 6) (Ridley *et al.*, 2005). Genes of flavin-dependent halogenases have only been found in the biosynthetic gene clusters for chloramphenicol and the neocarzilins, but not in the clusters for the other metabolites containing halogenated aliphatic groups, suggesting that a different type of halogenating enzymes might be involved in the chlorination of nonactivated aliphatic groups.

With all these genes of flavin-dependent halogenases identified, one wonders why *in vitro* activity has only been demonstrated for very few flavin-dependent halogenases. The answer lies in the nature of the substrates. Flavin-dependent halogenases that accept tryptophan as a substrate, monodechloroaminopyrrolnitrin 3-halogenase, a halogenase from the pentachloropseudilin producer *Actinoplanes* sp. ATCC 33002 (Wynands and van Pée, 2004), have been shown to catalyze the

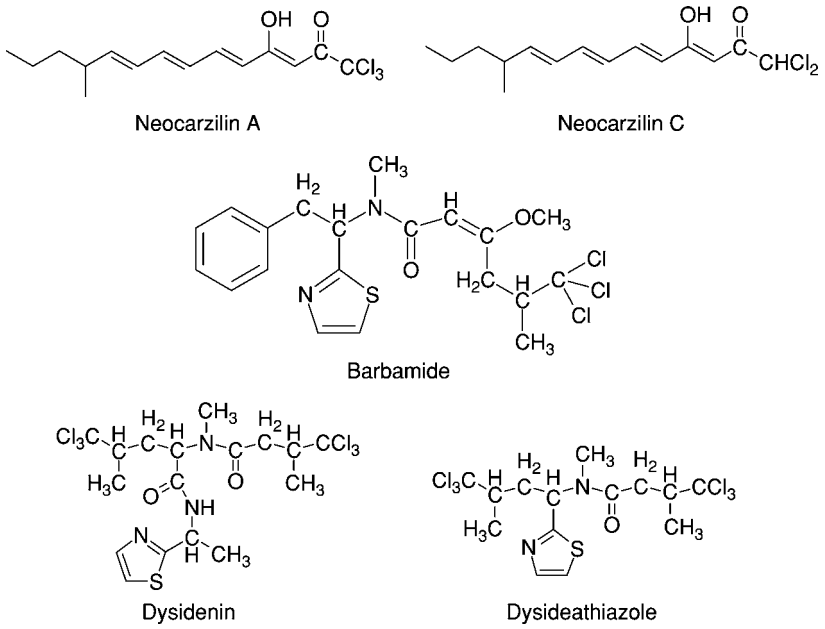


FIG. 6. Examples of halometabolites containing dichloromethyl or trichloromethyl groups.

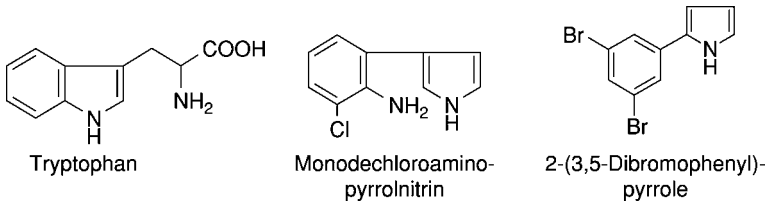


FIG. 7. Substrates which are accepted in their free form by halogenases.

chlorination of their substrates or substrate analogues in their free form (Fig. 7), but this might be the exception from the rule. Looking closely at the biosynthetic pathways for halometabolites, which have been identified either biochemically or by molecular genetic methods, it can be realized that it is very difficult to predict at which step in the biosynthesis halogenation occurs. To make things even more complicated, in polyketide and nonribosomal peptide synthesis the potential substrates are quite likely not the free molecules, but they are probably halogenated while they are either bound to an acyl or to a peptidyl carrier protein. Supplementation of a mutant of the balhimycin producer, *Amycolatopsis balhimycina*, blocked in the biosynthesis of *β*-hydroxytyrosine with 3-chloro-*β*-hydroxytyrosine did not restore balhimycin production, whereas supplementation with *β*-hydroxytyrosine did (Puk *et al.*, 2004). Since it can be assumed that during balhimycin biosynthesis *β*-hydroxytyrosine, after its original formation does not occur in free form anymore, but either as an acyl-AMP derivative or bound to a peptidyl carrier protein or other amino acids during formation of the heptapeptide backbone, chlorination of *β*-hydroxytyrosine can only take place while it is bound to another molecule. The study published by Dorrestein *et al.* (2005) on the chlorination reactions in pyoluteorin biosynthesis suggests that it might be the *β*-hydroxytyrosine-*S*-carrier protein form. For the halogenase PltA from pyoluteorin biosynthesis it could be shown that chlorination occurs while pyrrole-2-carboxylic acid, derived from proline, is bound to a pyrrolyl-*S*-carrier protein (Fig. 8). Obviously the halogenase does not accept free pyrrole-2-carboxylic acid, but also requires some features of the pyrrolyl-*S*-carrier protein for substrate recognition. If this is also the case for other flavin-dependent halogenases, it will be difficult to show their *in vitro* activity, since these substrates will not be easily available. Dorrestein *et al.* (2005) had to go a long way to obtain the substrate for PltA.

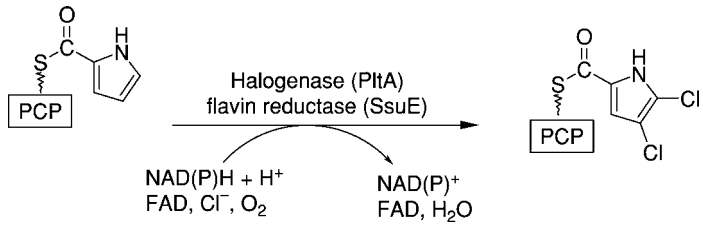


FIG. 8. Chlorination of pyrrol-2-carboxylic acid as an example for a halogenase substrate only accepted when bound to a peptide carrier protein (PCP).

C. Reaction Mechanism and Three-Dimensional Structure of Flavin-Dependent Halogenases

During the purification of the tryptophan 7-halogenase (PrnA) from pyrrolnitrin biosynthesis, it became evident that chlorination of tryptophan was actually catalyzed by a two-component system, consisting of the halogenase and a flavin reductase (Keller *et al.*, 2000). The flavin reductase catalyzes the formation of $FADH_2$, which is then presumably bound by the halogenase. The conservation of the GxGxxG motif in halogenases and flavin-dependent monooxygenases suggests a mechanistic similarity. In flavin-dependent monooxygenases, enzyme-bound $FADH_2$ reacts with oxygen to form FADH–OOH. This highly reactive electrophile hydroxylates aromatic substrates (Massey, 1994). The first hypothesis for the reaction mechanism of PrnA suggested a monooxygenase-like reaction of the FADH–OOH intermediate with the organic substrate tryptophan resulting in the formation of an epoxide. Nucleophilic attack by a chloride or bromide ion would then lead to halohydrin formation, and subsequent dehydration would result in the chlorinated end-product (Keller *et al.*, 2000). In this hypothetical mechanism, regioselectivity would be determined by the attack of FADH–OOH on the organic substrate together with the attack of the halide ion on the epoxide intermediate. The dehydration required in this mechanism would have to proceed in *cis*, which presents a major problem. Hubbard and Walsh (2003) suggested an electrophilic mechanism in which chloride would react with the FADH–OOH to form an FADH–OCl intermediate, which would then react with the organic substrate. Both hypothetical mechanisms were driven by a desire to avoid postulating free hypohalous acid, such as is seen in haloperoxidase, and perhydrolase-initiated halogenation reactions. As free hypohalous acid does not chlorinate tryptophan (Morrison and Schonbaum, 1976) and lacks regioselectivity, flavin-dependent

halogenases could not be producing free hypohalous acid. The three-dimensional structure of PrnA clearly showed that the protein is composed of two modules, an FAD-binding module and a tryptophan-binding module. The structure would seem to rule out any mechanism involving direct reaction of the organic substrate with FAD (Dong *et al.*, 2005). The FAD binding module of PrnA has significant structural similarity to *p*-hydroxybenzoate hydroxylase (Wierenga *et al.*, 1983), whereas the part that binds the organic substrate is novel. In the FAD-binding module of PrnA, there is no room for an organic molecule to bind adjacent to the isoalloxazine ring, instead the space at the isoalloxazine ring is occupied by two tryptophan residues (W272 and W274) (Fig. 9). This WxWxIP motif is conserved in flavin-dependent halogenases (Kling *et al.*, 2005).

The structure showed that flavin and tryptophan were lined by a 10-Å tunnel (Fig. 9). It also placed the chloride ion-binding site adjacent to the flavin. The chloride ion being nucleophilic would be in position to react with FADH–OOH. Since any flavin-bound electrophilic chloride species would not be able to make contact with the organic substrate, the formation of hypochlorite as a freely diffusible electrophilic chloride species is suggested (Fig. 10). Since hypochlorite by

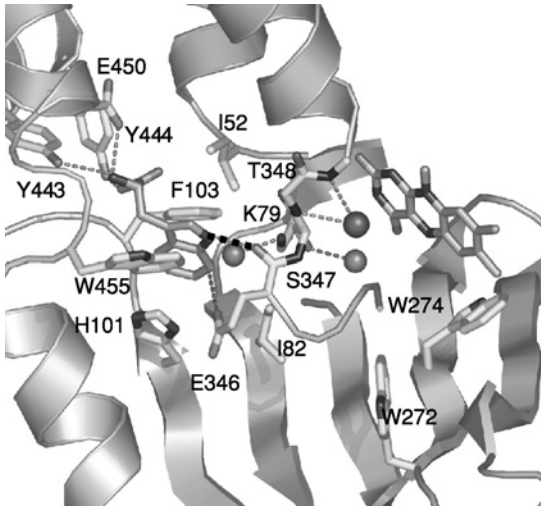
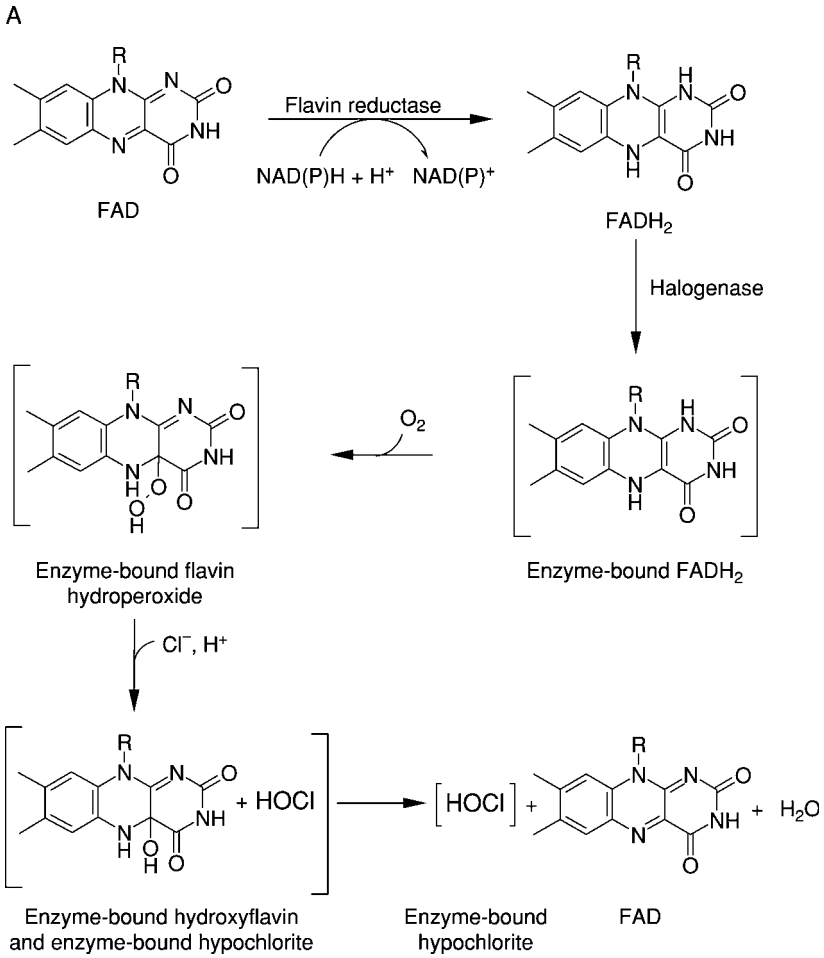


FIG. 9. Active site of tryptophan 7-halogenase (PrnA) from pyrrolnitrin biosynthesis. A tunnel leads from the flavin cofactor (on the right hand side of the figure) to the substrate tryptophan (trapped in between F103 and W455). Chloride (shown as the larger sphere) is bound close to flavin cofactor. The distance between the flavin cofactor and the substrate tryptophan of about 10 Å prevents their direct contact. Two water molecules are shown as spheres.

itself would not be able to chlorinate tryptophan, its electrophilicity must be enhanced. This could be done by interaction of the OH^- of hypochlorite with the amino group of lysine 79 which is ideally positioned about halfway between the flavin and the organic substrate. This lysine residue could enhance the electrophilicity of the chloride species by protonation or alternatively form a chloramine. In either case, guide it to the correct position at the organic substrate. The requirement for a lysine residue would prevent hypochlorite from reacting with other



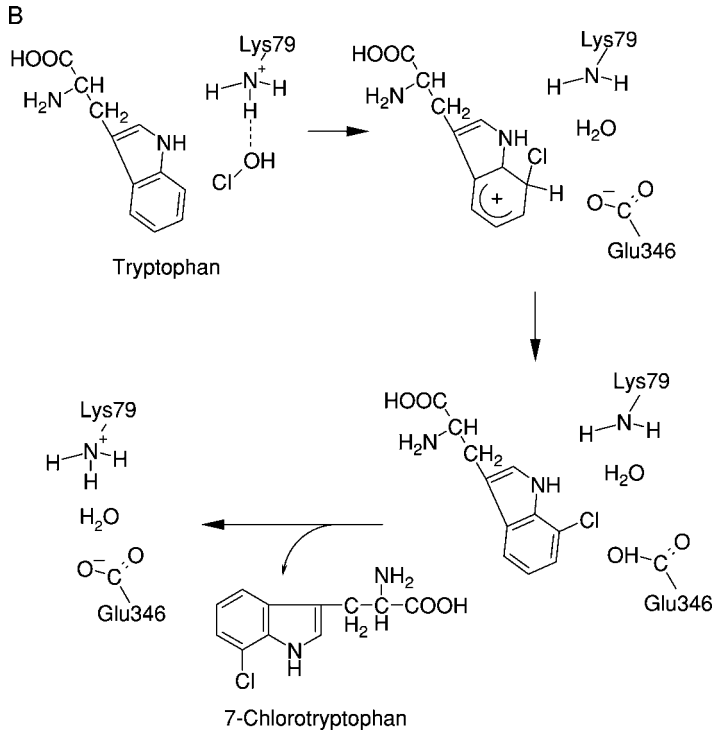


FIG. 10. Proposed reaction mechanism of flavin-dependent halogenases. (A) In the first part of the reaction FAD is reduced to $FADH_2$ by a flavin reductase. (B) In the second part of the reaction HOCl is formed at the active site of the enzyme and is guided by interaction with lysine 79 to the correct position at the substrate tryptophan. Flavin blocks the entrance to the tunnel thus preventing HOCl from leaving the active site.

amino acid residues in the active site. Rearomatization of the benzene ring of tryptophan after reaction with the Cl^+ species requires abstraction of a proton. Glutamate 346 is ideally positioned to do this (Figs. 9 and 10). The formation of hypochlorite at the active site would explain why indole derivatives, such as 5-methylindole or indole-3-acetonitrile, are not chlorinated by PrnA in the 7-position, but rather at the 2- and/or 3-position of the indole ring. These are the positions that are chlorinated by free hypochlorite in solution. Evidence for the proposed mechanism comes from a series of mutants in which lysine 79 is exchanged for alanine or methionine, and glutamate 346 is changed to glutamine. In each case the mutant is either inactive or 100-fold less active than the native enzyme (Dong *et al.*, 2005).

IV. α-Ketoglutarate–Dependent Halogenases

Chlorination initiated by haloperoxidases and perhydrolases as well as the halogenation reactions catalyzed by flavin-dependent halogenases require a substrate with a double bond. From labeling studies during the investigation of barbamide biosynthesis in the marine cyanobacterium *Lyngbya majuscula* it is known that leucine is a precursor of the trichloromethyl group in barbamide (Fig. 6). It was shown that the hydrogen atom at C4 of leucine is never removed during barbamide biosynthesis and thus a double bond between C4 and any of the two methyl groups cannot be formed (Sitachitta *et al.*, 2000). Thus, the involvement of a flavin-dependent halogenase in barbamide biosynthesis is ruled out and already quite early a radical mechanism was suggested for the chlorination of the leucine-derived methyl group (Sitachitta *et al.*, 1998). When Chang *et al.* (2002) cloned the barbamide biosynthetic gene cluster, they could not find a gene coding for an enyzme with any similarity to already-known halogenating enzymes. They only detected two genes (*barB1* and *barB2*) with similarity to the genes coding for SyrB1 and SyrB2 from the syringomycin E biosynthetic gene cluster from *Pseudomonas syringae* (Guenzi *et al.*, 1998). However, the function of Barb1/Barb2 and SyrB1/SyrB2 as halogenases could not be shown at that time. Genes (*dysB1/dysB2*) similar to the *barB1/barB2* and *syrB1/syrB2* pairs were detected in *Oscillatoria spongeliae* strains, cyanobacterial symbionts of dictyoceratid sponges, producing dysidenin and dysideathiazole (Ridley *et al.*, 2005) (Fig. 6).

Proof that SyrB2 was actually a halogenase was presented by Vaillancourt *et al.* (2005a). Based on the observation that the SyrB2 and Barb2 amino acid sequences predicted that they could be nonheme Fe^{II}, α-ketoglutarate–requiring enzymes halogenating activity could be shown *in vitro* for SyrB2. But like in the case of flavin-dependent halogenases, the substrate specificity of the enzyme made demonstration of the enzymatic activity difficult. The enzmye did not accept free threonine. Chlorination of threonine was only catalyzed by SyrB2 when threonine was bound to the phosphopantetheinyl group of the thiolation domain of SyrB1 (Fig. 11A). SyrB2 is an oxygen-labile Fe^{II} enzyme that requires α-ketoglutarate and oxygen for its chlorinating activity. The reaction mechanism suggested involves the formation of a substrate radical by abstraction of an H-radical by the Fe^{IV}═O intermediate which then abstracts a Cl-radical from the Fe^{III}–OH intermediate (Fig. 11B).

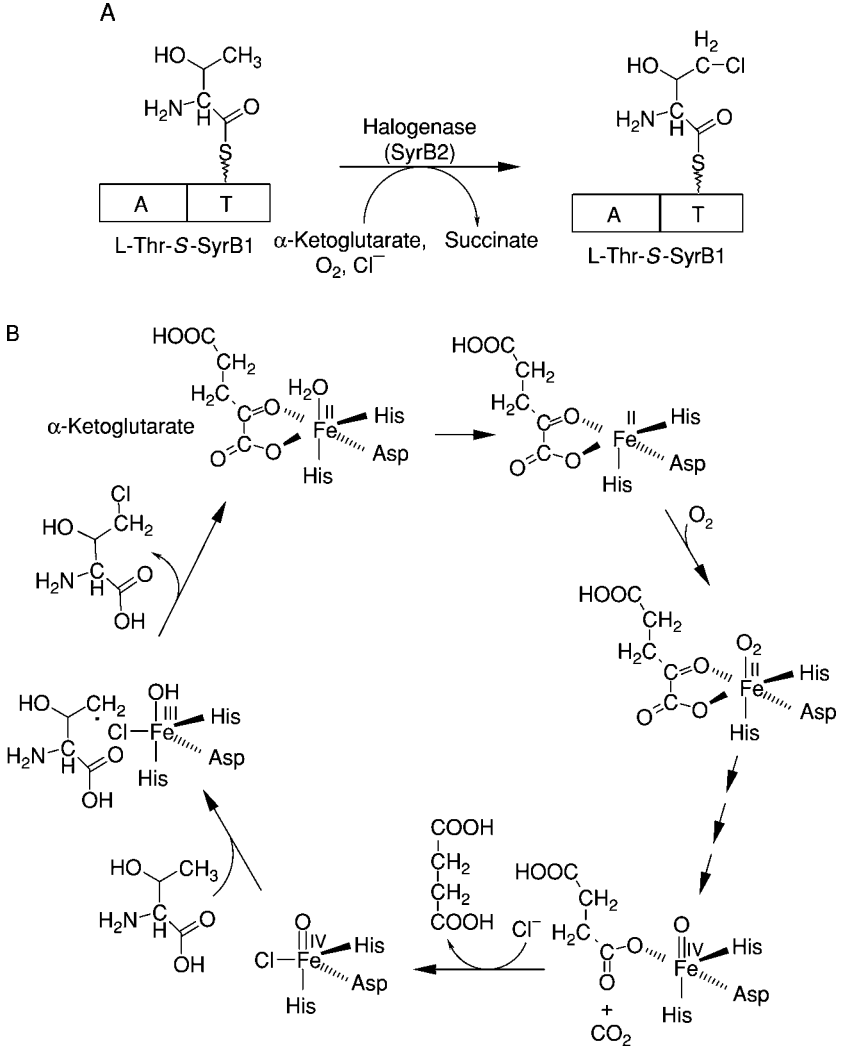


FIG. 11. Chlorination of a nonactivated alkyl group by a nonheme Fe^{II}, α-ketoglutarate- and oxygen-dependent halogenase. (A) Previous to halogenation, the substrate is bound to a thiolation domain. (B) In the suggested mechanism, a substrate radical is formed which attacks the chlorine bound to the nonheme iron.

Another example for this novel type of halogenases is CmaB involved in the formation of coronamic acid from L-*allo*-isoleucine as an intermediate in coronantine biosynthesis (Vaillancourt *et al.*,

2005b). Again the halogenating enzyme does not accept its substrate in a free form but requires the substrate to be tethered to CmaD, a standalone 8-kDa thiolation domain. It can be assumed that Barb2 and DysB2 also belong to this new class of nonheme Fe^{II}, α-ketoglutarate- and oxygen-dependent halogenases and Barb1 and DysB1 function as thionylation domains, necessary for binding the substrate of the halogenases and for their substrate recognition.

V. Fluorinase

Streptomyces cattleya produces fluoroacetic acid and 4-fluorothreonine (Sanada *et al.*, 1986). Feeding experiments with labeled potential precursors suggested that glycerol (Tamura *et al.*, 1995) or glycolate (Reid *et al.*, 1995) could be a precursor for fluoroacetate biosynthesis. Tamura *et al.* (1995) speculated that fluorination might occur by displacement of the hydroxyl group of *β*-hydroxypyruvate, an intermediate derived from glycerol by fluoride, whereas Reid *et al.* (1995) suggested displacement of the phosphate group of phosphoglycolate by fluoride as the fluorination step. It was clear that fluorination could not be catalyzed by a haloperoxidase or perhydrolase type of enyzme, since oxidation of fluoride using hydrogen peroxide is not possible. Thus fluorination had to proceed by nucleophilic attack of the fluoride ion. Zechel *et al.* (2001) constructed mutants of two glycosidase enzymes, which create a very potent electrophilic substrate that can be attacked by fluoride ion which in aqueous solution is only a very poor nucleophile. To allow carbon–fluorine bond formation, either the electrophilicity of the substrate has to be enhanced or the nucleophilicity of the highly hydrated fluoride ion has to be increased by dehydration. This is achieved by the fluorinase detected in the fluoroacetate producer (Schaffrath *et al.*, 2002). The identification of *S*-adenosyl-L-methionine as the substrate for the fluorinase was quite unexpected. The reaction proceeds by replacement of L-methionine by fluoride resulting in the formation of 5′-fluoro-5′deoxyadenosine, which is further metabolized to 4-fluorothreonine and fluoroacetate via fluoroacetaldehyde (Fig. 12) (Murphy *et al.*, 2001). Crystallization and elucidation of the three-dimensional structure of fluorinase revealed that the reaction probably proceeds by an S_N2 mechanism. The structure suggests that the fluoride ion is fully dehydrated by the enzyme, enhancing its nucleophilicity (Dong *et al.*, 2004). This unusual enzyme is so far the only natural fluorinating enzyme.

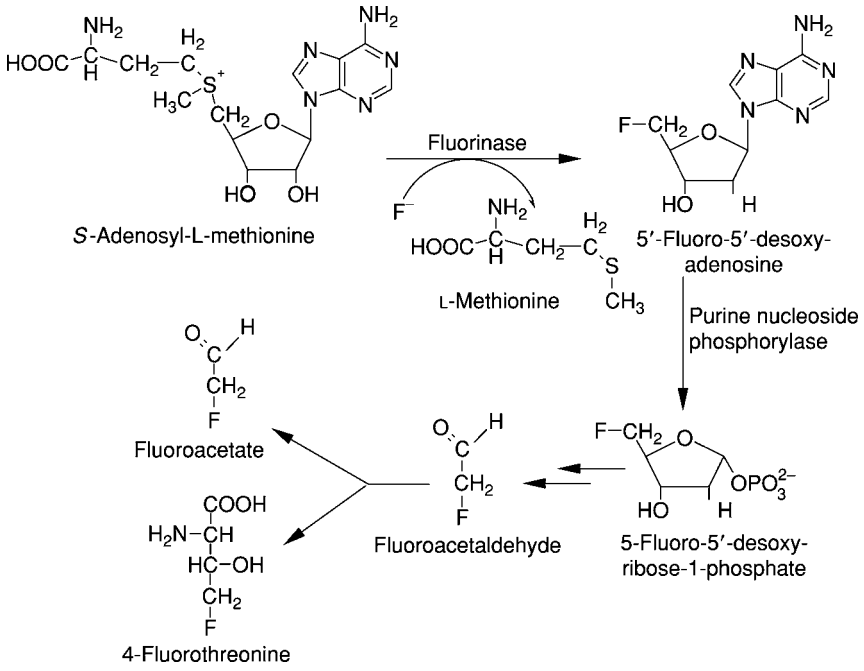


FIG. 12. The fluorinase reaction leading to the formation of 5′-fluoro-5′-desoxyadenosine from *S*-adenosyl-L-methionine as the substrate and further metabolization of 5′-fluoro-5′-desoxyadenosine to fluoroacetate and 4-fluorothreonine.

VI. Methyl Transferases

Another type of halogenating enzymes catalyzing the incorporation of halide ions into organic molecules as a nucleophile are a specific type of methyl transferases (Saxena *et al.*, 1998; Wusomaa and Hager, 1990). These methyl transferases use, like the fluorinase, *S*-adenosylmethionine as the organic substrate. They transfer the methyl group from *S*-adenosylmethionine to chloride, bromide, or iodide (Fig. 13). Methyl transferases using halide ions as the halide acceptor have been detected in marine algae, fungi, and terrestrial and marine bacteria (Amachi *et al.*, 2001; Harper *et al.*, 1989; Wusomaa and Hager, 1990).

VII. Halogenating Enzymes in Biotechnology

Intensive investigations into the use of haloperoxidases and perhydrolases have been made by many groups (Neidleman and Geigert,

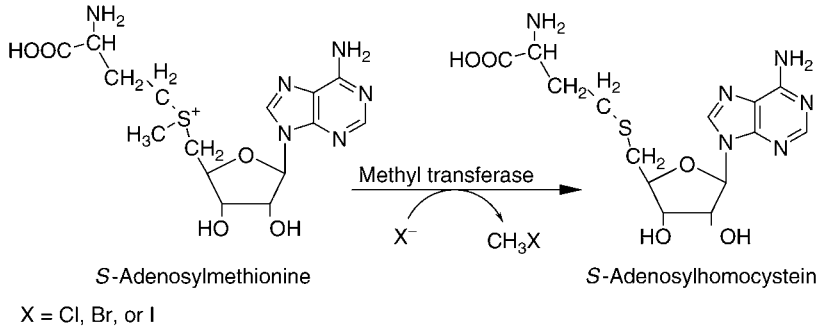


FIG. 13. Formation of methyl halides from *S*-adenosylmethionine catalyzed by methyl transferases.

1986). However, the outcome was altogether rather disappointing. Haloperoxidases had no advantage over chemical halogenation reactions with regard to regioselectivity, stereospecificity, and by-products (van Pée, 1996). Nowadays this can easily be explained by the formation of free hypohalous acids by these types of halogenating enyzmes.

The novel flavin-dependent halogenases are highly superior to haloperoxidases due to the fact that halogenating reactions catalyzed by these enyzmes proceed with regioselectivity and without the formation of by-products. However, their use in biotechnology in *in vitro* reactions might be hampered by the requirement of a flavin reductase and NADH and FAD as cofactors. While FAD is only required in very low concentrations (10 μM) and is regenerated during the reaction, NADH has to be used in much higher concentrations (2.5 mM). Although the reductase/NADH system can be substituted by an organometallic catalyst using formate as the electron donor for FAD reduction, at least the activity of tryptophan 7-halogenase from pyrrolnitrin biosynthesis is strongly inhibited by the formate concentrations required by the organometallic catalyst (Unversucht *et al.*, 2005).

The use of the α-ketoglutarate–dependent halogenases as a catalyst in *in vitro* reactions seems even more problematic, since these enzymes are oxygen-labile (Vaillancourt *et al.*, 2005a). Another severe problem for the use of halogenases in biotechnology lies in their high-substrate specificity, especially when only substrates tethered to carrier proteins are accepted for halogenation.

However, both types of halogenases, the flavin-dependent and the α-ketoglutarate–dependent ones, might be very suitable for the production of halometabolites *in vivo*, especially in combinatorial biosynthesis.

First attempts to use the genes of flavin-dependent halogenases for the formation of novel halometabolites by introducing tryptophan halogenase genes coding for tryptophan halogenases with a different regioselectivity into the rebeccamycin producer *Lechevalieria aerocolonigenes* lead to the formation of novel halogenated metabolites (Sánchez *et al.*, 2005). A similar approach was used by Eustaquio *et al.* (2004) who obtained novel derivatives of novobiocin by introducing the halogenase gene from clorobiocin biosynthesis together with the novobiocin gene cluster from *Streptomyces spheroides* into *Streptomyces coelicolor*.

These first results have been very promising, and the next few years will certainly see many new results on the use of the new halogenating enzymes and their genes for the formation of novel halogenated compounds.

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Phage for Rapid Detection and Control of Bacterial Pathogens in Food

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I. Introduction

In recent years there has been a revival of interest in the use of phage to treat bacterial infections, and research using phage therapy to overcome the problem of increasing levels of antibiotic resistance has become widely publicized (Sulakvelidze and Kutter, 2005). Accordingly, the idea that phage could be applied to food products as biocontrol agents has also received more interest among researchers. However, those working in the dairy industry are only too aware of the potential for these viruses to destroy populations of bacteria in milk-starter cultures. On the other hand, the use of phage is nothing new in the field of bacterial characterization, and phage typing schemes are routinely used in the subtyping of isolates of organisms such as *Salmonella enterica* (Anderson *et al.*, 1977; Laszlo and Csorian, 1988), *Staphylococcus aureus* (Blair and Williams, 1961), *Listeria monocytogenes* (Rocourt, 1996), *Vibrio cholerae* (Basu and Mukerjee, 1968), and *Escherichia coli* O157:H7 (Khakhria *et al.*, 1990). Over the last 10 years much work has been carried out to develop phage-based methods for rapid detection of

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 59 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(06)59006-9

pathogens in foods and, although the only commercially available test is that for detection of *Mycobacterium tuberculosis* in human sputum samples (Mole and Maskell, 2001), many new tests and applications for detection of pathogens in foods are currently being developed. This chapter will provide an overview of the recent developments in these fields and look at some new areas that may be developed into practical applications in the future.

II. Phage Typing

The ability of a phage to infect a particular cell will be dependent on variation in primary surface receptors, which may comprise surface polysaccharides (lipopolysaccharide in Gram-negative bacteria, Eriksson, 1977; the teichoic acids in Gram-positive bacteria, Estrela *et al.*, 1991), the presence of surface structures (such as flagellae or pili; Iino and Mitani, 1966; Joys, 1965; Merino *et al.*, 1990; Romantschuk and Bamford, 1985), and the expression of a wide range of different types of cell-surface–associated molecules including sugar uptake proteins (Schwartz, 1983), membrane proteins (Heilpern and Waldor, 2000; Sun and Webster, 1987), S-layer proteins (Callegari *et al.*, 1990), and capsular polysaccharide (Hung *et al.*, 2002). Even if phage successfully penetrate the cell envelope, replication leading to cell lysis and development of a plaque may be inhibited by a variety of mechanisms, such as the presence of prophage in the cell (Harvey *et al.*, 1993), DNA restriction-modification systems (Frank, 1994), and even specific phage inhibition genes (Chopin *et al.*, 2005). These many factors which affect the efficiency of phage replication have lead to the development of phage-typing schemes, where the ability of a phage to infect a cell is used as an indicator of biological variation at the cellular level.

For phage typing, a panel of phage characterized to have a limited host range are chosen and strains are infected with each phage at standard concentration (known as the Routine Test Dilution or RTD). Hence, a bacterial lawn is prepared and samples of the different phage at the RTD spotted onto the surface of the lawn. After incubation, infection is detected by the presence of plaques (zones of clearing) and patterns of susceptibility to individual phage are determined, leading to the characterization of a phage type (Fig. 1). These panels of phages have successfully been used by epidemiologists to monitor changes in the predominant organism causing disease in the population and to identify the emergence of new dominant clones. For *Salmonella*, in particular, the phage-typing system (Callow, 1959) is successfully used to further differentiate strains of the same serovar, as

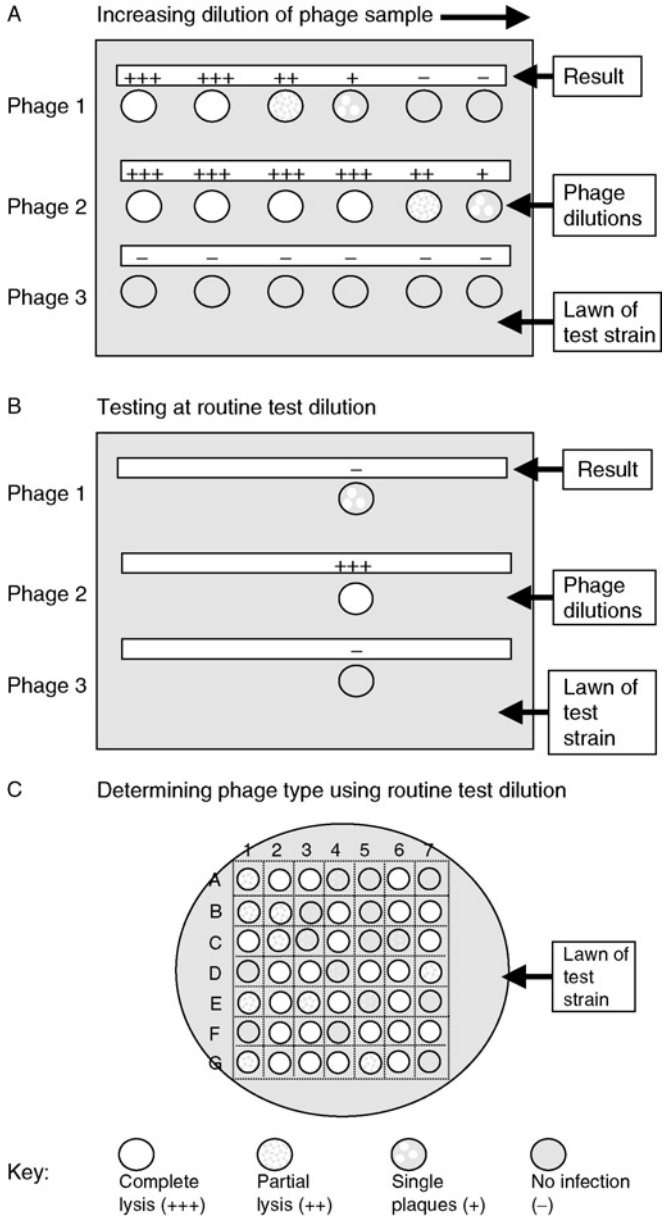

FIG. 1. Phage typing: For a given phage, each host strain will allow replication with a specific efficiency (efficiency of plating or e.o.p). To determine this value, a lawn of the host strain is prepared by inoculating it at 10–10^7 cfu ml^{-1} into a molten soft agar, and

the widespread nature of some serovars requires more discriminatory methods for subtyping than is afforded by serology alone (McDonough *et al.*, 1989; Threlfall *et al.*, 1978a,b, 1994). The use of detailed subtyping has allowed sources of foodborne disease to be identified. For instance, this technique has been successfully used to identify the emergence of clones that come to predominate in food industries so that new risks and intervention strategies can be established. For example, the emergence of *Salmonella enteritidis* PT4 in poultry flocks was seen to be the cause of outbreaks from the mid 1980s until recently and led to a change in advice to the public to avoid consumption of raw eggs and also the implementation of a culling program to eradicate the organism from breeding flocks. Similarly, when rare phage types emerge (often through importation of foods), phage typing can greatly assist in the identification of outbreak sources. In 2003 *Salmonella enteritidis* PT 56, rare in the United Kingdom, caused an outbreak in the northeast of England, which was then traced back to a particular restaurant and workers in that restaurant were found to be carrying the strain responsible for the outbreak (Anon, 2004).

III. Rapid-Detection Methods

Phage typing, however, is not a rapid method and strains can only be typed after they have been isolated and identified. The fact that phage replicate far faster than their host cells has lead to their use in a variety of rapid detection methods for bacterial pathogens. Given this background of using phage with a limited host range for replication to subtype bacteria, it seems surprising that phage can be used to develop generic detection methods for bacterial pathogens. However, it must be

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this is poured over a solid base agar and allowed to set. Dilutions (normally 10-fold) of the phage to be tested are prepared and samples of each dilution spotted onto the lawn. Plates are incubated under optimal conditions for phage infection until the lawn has grown to stationary phase. The e.o.p. is determined by comparing the number of plaques per ml detected on a lawn of this host strain with the number determined for the same phage preparation on propagating strain for that phage (panel A). Using multiple dilutions of phage for each phage-typing test is not practicable, therefore a single standard dilution of phage is chosen as the Routing Test Dilution (RTD). To allow for experimental variation, a cut-off value is applied and low numbers of plaques are scored as negative (panel B). A panel of phage with different lytic spectra are assembled; phage included in the panel will ideally not infect all isolates of the bacterium. Finally, an RTD sample of each of the phage in the panel used is applied in a set grid pattern to a lawn of the test bacterium. Results are scored and a phage sensitivity pattern (or phage type) is established.

remembered that those phage chosen for phage-typing panels are specifically chosen for their limited host range because the infection event is sensitive to differences in cell structure.

When developing a test to detect whole genera or species, phage with the widest host range are chosen. One of the earliest examples of this is the *Salmonella* phage Felix 01, described as a broad host range phage, which could be used to detect the presence of *Salmonella enterica* by Cherry *et al.* (1954), and more recent studies have shown that it will usually infect more than 95% of *Salmonella* isolates (Kuhn *et al.*, 2002). Similarly, Loessner and Busse (1990) described a listeriaphage (A511) that infected 95% of *Listeria* serotypes 1/2a and 4b, which are most commonly associated with human disease and has been included in identification schemes for this organism (Barbalho *et al.*, 2005). A survey of the published literature shows that such broad host range phage can be readily isolated, especially from those environments where the host cells are naturally found (Barman and Majumdar, 1999; Jensen *et al.*, 1998; Sullivan *et al.*, 2003).

A. Labeled Phage

Goodridge *et al.* (1999a) devised a phage-detection method using fluorescently labeled phage to detect *E. coli* O157:H7 in broth. The DNA of phage particles was stained with a fluorescent nucleic acid dye, YOYO-1, and these phages were used to infect *E. coli* O157:H7 cells, which had been captured by IMS. Here the phage are simply acting as a specific reagent to dye the surface of the infected cell in the same way as a labeled antibody is used to detect cells in an ELISA assay. Cells could be detected reliably at a level of 10^4 cells ml^{-1} by fluorescence using flow cytometry. The phage used, LG1, was selected to be specific for this serovar of *E. coli* with minimal cross reaction outside of the group but some cross reaction with non-O157:H7 cells was recorded. However, the specificity of the antibody used in the IMS separation meant that the only cells present in the sample to be infected with the labeled phage were of the correct serovar and the combination of two specific selection steps (antibody specificity and phage host range) meant that the overall specificity of the test was increased. When used to detect *E. coli* O157:H7 cells in food products (Goodridge *et al.*, 1999b), detection of 2.2 cfu g^{-1} could be achieved in artificially contaminated ground beef following preincubation of samples in BHI broth for 6 hours at 37°C, while for raw milk samples between 10^1 and 10^2 cfu ml^{-1} could be detected following a 10-hour preincubation step.

B. Reporter Phage

The idea that phage could be used to introduce a reporter gene into a bacterial cell had been well established by the development of phage Mud-*lac* as a genetic tool (Castilho *et al.*, 1984). Here, a promoterless reporter gene (β-galactosidase gene; *lacZ*) was introduced into the Mu bacteriophage. This phage inserts randomly into the host genome following infection and when insertion occurs next to a promoter sequence the *lacZ* gene is expressed. In this case, the reporter gene expression is monitored by the hydrolysis of a chromogenic substrate (X-gal) giving a blue coloration that is detected spectrophotometrically. However, only some infected cells have this phenotype (i.e., when the phage integration occurs next to a chromosomal promoter). In true reporter phage assays, the reporter gene is expressed from a promoter within the phage so that all infected cells express the reporter gene and produce the detectable phenotype.

Ulitzur and Kuhn (1987) first developed the idea of using the expression of the reporter genes following phage infection, specifically as a rapid method of bacterial detection. Originally they used simple phage-lambda cloning vectors (Charon 30) containing the bacterial bioluminescence (*lux*) operon, and using such constructs it was shown that as few as 10 *E. coli* cells could be detected within 1 hour in milk samples (Ulitzur and Kuhn, 1989). They also used random mini Tn*10-luxAB* mutagenesis of wild-type phage genomes and selected functional phage that could replicate and produce bioluminescent plaques. These constructs have been shown to successfully detect the presence of enteric pathogens in food samples. Kodikara *et al.* (1991) used such recombinant *lux* phage to detect enteric bacteria from swabs of abattoir, meat-processing factory surfaces, and carcasses. This method showed good correspondence between bioluminescence levels and viable count and allowed detection of 10^4 cfu g^{-1} or cm^{-2} within 1 hour; at levels <10 cfu g^{-1} or cm^{-2}, detection could be achieved in 1 hour following a 4-hour enrichment. The value of the approach as a method for hygiene monitoring was evident, detecting a high-level contamination event occurring at a site in the abattoir within 1 hour of sampling.

Another example of random mutagenesis being successfully used to generate reporter phage is described by Waddell and Poppe (2000). Again a mini Tn*10* transposon was used to randomly insert the *luxAB* reporter genes into the genome of a phage specific for *E. coli* O157:H7 (NV10; Khakhria *et al.*, 1990). In this case, the size of the insertion was larger than the packaging constraints of the phage head and the phage were not able to replicate, however, they were able to detect *E. coli*

O157:H7 cells within 1 hour of infection in a microtiter plate assay through the expression of the *lux* genes once introduced on the phage genome.

An alternative approach to generate *lux* reporter phage is described by Chen and Griffiths (1996). This group used homologous recombination to introduce the *luxAB* genes into phage either by infecting *Salmonella* strains carrying the *luxAB* genes on plasmids with phage P22, or by induction of lysogenic phage from similar lux^+ cells. A cocktail of these phage was used that targeted groups B, D, and some group C serovars and allowed detection of 10^8 cfu ml^{-1} in 1 to 3 hours with no preincubation; as with Kodikara *et al.* (1991) as few as 10 cfu ml^{-1} of *Salmonella* cells in the original sample were detectable with 6 hours of preincubation. An interesting feature of this study was the demonstration that phage could be used to show the presence of the *Salmonella in situ* inside the egg; phage were applied to *Salmonella*-infected eggs and bioluminescence imaging used to locate the areas of infection.

Ulitzur and Kuhn (1989) chose to insert only the *luxAB* genes (2.1 kbp) into the phage so that they did not exceed the packaging constraint of the phage genome. In the case of the Mud-*lac* phage, however, a compensatory deletion of genes is required to allow the insertion of the *lacZ* gene (Castilho *et al.*, 1984). This is only possible if there is sufficient knowledge of the phage genomic organization so that essential genes are not deleted. Wolber and Green (1990a,b) used the well-characterized phage P22 as a basis of a *Salmonella* detection assay by introducing the ice nucleation gene (*ina*). The ina^+ phage allowed detection of very low levels of *Salmonella* due to the sensitivity of the reporter gene assay used (BIND assay: bacterial ice nucleation detection; Wolber, 1993), which is based on the color change of a fluorescent dye when samples are cooled. This assay allowed the detection of samples containing only 2 cells ml^{-1} *Salmonella enteritidis* in a buffer system or in raw egg within 3 hours. It was also able to detect 10 *Salmonella* Dublin cells ml^{-1} in the presence of a high level of competitor organisms, removing the need for selective enrichment steps prior to detection. The sensitivity of the assay for food samples was further increased by using it in combination with salmonellae-specific immunomagnetic bead separation (Irwin *et al.*, 2000). This assay was developed commercially by the Idetek Corporation but is no longer marketed.

Kuhn *et al.* (2002) have taken this approach to the extreme and, following extensive genome characterization of phage Felix-01, created a ''locked'' phage that not only contained compensatory deletions of nonessential regions but also contains mutations that make the phage

defective when replicating in wild-type cells. Hence following infection of *Salmonella* cells in samples, the luciferase genes are successfully expressed, allowing detection of the infected cell, but no viable phage particles are produced at the end of the infection cycle. This strategy was employed to tackle concerns raised about the release of GM phage into the environment, however, useful tests have been developed and marketed without the requirement to meet this stringent level of genetic containment.

A more direct and less cumbersome strategy is to insert the reporter gene into a phage operon so that all phage-essential functions are retained. The strategy is generically applicable to all phage and requires a minimum amount of genome analysis. The packaging constraints of the individual phage still need to be met, but any phage with a genome of greater than 50 kbp is likely to be able to accommodate most of the commonly used reporter genes (firefly luciferase (*luc*) 2.3 kbp; bacterial ice nucleation protein (*ina*) 3.4 kbp; green fluorescent protein (*gfp*) 0.8 kbp, β-galactosidase (*lacZ*) 1.1 kbp; Goodridge and Griffiths, 2002).

Loessner *et al.* (1996) used this approach to introduce the *luxAB* genes into the broad-host range listeriaphage A511. Phage structural genes were cloned and sequenced and then the *luxAB* genes inserted into the phage genome by recombination, creating an operon fusion without disrupting any of the original gene structures. An evaluation of the ability of the A511 reporter phage to detect *Listeria monocytogenes* was carried out in a range of food samples comprising artificially and naturally contaminated meat, fish, dairy, and vegetables together with environmental samples from dairy plants (Loessner *et al.*, 1997). A short (20 hours) selective enrichment stage in *Listeria* enrichment broth was used prior to the phage assay giving a total assay time of 24 hours. From this study it was clear that, as for many rapid methods applied to food systems, the nature of the food matrix and the competing microflora is critical to the sensitivity of the test. In food samples with a low background flora level (ricotta cheese, chocolate pudding, and cabbage), detection was possible from an initial inoculant level of 0.1 cells g^{-1}, while in foods with a large endogenous microflora (minced meat and soft cheese) an initial inoculant level of 10 cells g^{-1} was needed. This study highlighted two of the main advantages of phage-based tests; the specificity of the host-phage interaction allows the detection of low numbers of specific bacteria in a mixed population without the need to purify to homogeneity by successive rounds of enrichment and selective plating. Hence the test time can be reduced to 24 hours, even with enrichment incubation. It also has the advantage over DNA and antibody-based tests

that it only detects viable cells, and this is of particular significance where heat treatment or other cell-inactivation methods have been applied as part of the production process.

More recently, the same method has been used to generate a *gfp* reporter phage for the detection of *E. coli* O157:H7 using phage PP01 (Morita *et al.*, 2002). Again, structural genes were identified and the *gfp* gene introduced into the phage genome by homologous recombination so that no native phage genes were disrupted (Oda *et al.*, 2004). This phage was able to specifically detect *E. coli* O157:H7 cells in the presence of other competitive *E. coli* cells but the limits of detection were not reported. However the authors showed that the phage could be used to detect both actively growing and dormant cells that may be of use to those working on sublethally injured cells or for detection of *E. coli* O157:H7 cells in water samples. In a further development of this work, the *gfp* gene was introduced into a mutant of phage T4 that is unable to lyse the *E. coli* host cells at the end of the replication cycle. Here, the infection of the cells leads to sustained expression of the *gfp* gene without cell lysis, effectively labeling the cells with Gfp protein (Tanji *et al.*, 2004).

Another reporter phage for *E. coli* O157:H7 has been produced by workers at the University of Wyoming, USA (Dr L. Goodridge, personal communication). Here the *lacZ* gene has been introduced into phage T4 and the reporter phage has been combined with an enrichment broth, immunomagnetic beads, and an enzyme substrate to produce a simple swab-based test called Phast Swab. After swabbing the test surface, the swab is placed into the sample tube and an 8-hour incubation is carried out. All *E. coli* cells present are concentrated using the IMS beads and finally any viable *E. coli* O157:H7 cells are detected by infecting with the reporter phage. When a luminescent substrate is used, between 10^2 and 10^3 *E. coli* O157:H7 cells can be detected within 12 hours. The advantage of this test is that it has been formatted to be user friendly with a minimum number of manipulations and does not require the purchase of expensive, specialized detection equipment since luminometers are routinely found in many food production environments as part of standard hygiene-testing regimes (Section III).

C. Use of Phage in Other Rapid Methods

Development of the reporter phage assays, however, is slow and costly, as both phage characterization and genetic engineering of phage is required and means that new phage cannot be developed quickly for new genera or new species/subspecies. For a reporter phage test to be

adopted, there must be some obvious advantage over conventional microbiological tests. A method of rapid detection that can be easily adapted to detect these new groups is the Phage Amplification technology that uses only wild-type phage since the endpoint of detection is the formation of a plaque. Stewart *et al.* (1998) demonstrated the versatility of the method in that they simultaneously developed assays for the detection of *Pseudomonas aeruginosa, Salmonella typhimurium*, and *Staphylococcus aureus*. Detection of *Mycobacterium tuberculosis* using this technique has also been described (McNerney *et al.*, 1998; Stewart *et al.,* 1992; Wilson *et al.*, 1997) and a Phage Amplification assay is marketed commercially for the detection of *Mycobacterium tuberculosis* in human sputum samples (FAST*Plaque*TB™, Mole and Maskell, 2001). It is in the detection of these slow-growing pathogens that the test provides its biggest advantage. The signal that a pathogen is present is the replication of the phage. First, the test sample is mixed with phage to allow any target cells present to be infected. Then a virucide is added to destroy all those phage that have not successfully infected a bacterial cell, so phage only survive if they are protected inside a suitable host cell. The next stage is to neutralize the virucide, mix the sample with a laboratory phage-propagating strain (termed the ''helper'' bacteria), and then plate the whole sample in a soft agar overlay. Once the replication of the phage inside the infected test cell is complete, cell lysis of the target bacterium occurs, and the released phage infect cells of the laboratory phage propagating strain, forming plaques in the bacterial lawn. Hence each plaque represents the presence of one target bacterium in the original sample, which protected a phage from the virucide. Again, like the reporter phage assays, target cells must be viable to allow phage replication so the test is able to distinguish between live and dead cells.

As *M. tuberculosis* is a slow-growing organism, this rapid method provides an advantage in that the detection test only takes 2 days, compared to several weeks for other methods of detection. To achieve this rapid detection, the phage used has a broad host range and not only infects slow-growing *M. tuberculosis*, but also the fast-growing *Mycobacterium smegmatis*, which is used as the detection bacterium, hence the lawn develops and plaques can be visualized after overnight incubation. In this case specificity is achieved not because of the phage host range, but because of the nature of the sample tested; plaques may be due to any of the pathogenic Mycobacteria that can infect lungs, but only pathogenic mycobacterial cells will be present in human sputum samples so a positive result indicates infection and a need to treat the patient.

We have shown that the reagents from the FAST*Plaque*TB™ assay can be used for the detection of viable *Mycobacterium paratuberculosis* or *Mycobacterium bovis* in milk samples. Here the issue of specificity is again limited by the sample taken; milk samples should not contain large numbers of mycobacterial cells and those present are likely to be one of these two pathogens. To confirm the identity of the cell in the milk sample giving rise to the plaque, DNA was extracted from the plaque and then analyzed by PCR for the presence of signature IS elements (IS900 for *Mycobacterium paratuberculosis* and IS1081 for *Mycobacterium bovis*). This combined Phage Amplification PCR assay has allowed us to detect viable *Mycobacterium paratuberculosis* in milk samples from infected cattle within 24 hours (Stanley, 2005) (Fig. 2).

For fast growing bacterial pathogens there is no obvious advantage to the technique, as colonies can develop using standard microbiological detection methods within 12 hours. However this does not take account of the time required to carry out the confirmatory tests that are often needed following the presumptive identification of a bacterial colony. When developing the *M. paratuberculosis* assay, another advantage over standard methodologies was the fact that no selective enrichment was required which enhanced the detection levels and reduced the time required for the assay. In essence, we used the Phage Amplification technique as a single cell separation and DNA extraction method that simultaneously removed the inhibitory components of the food matrix from the sample confirmed the viability of the cell and gave some information about the identity of the cell detected as it was able to protect and propagate the phage. However, the certainty of the endpoint PCR-based DNA identification technique confers absolute specificity on this combination of tests, and future applications may be developed for fast-growing pathogens where separation of the bacteria from the food matrix and the time take for the confirmatory tests are the difficult and time-consuming aspects of a current assay.

A variation of the Phage Amplification assay has been described by Favrin *et al.* (2001). In this case, target cells are separated from the sample by immuno-magnetic separation using magnetic beads coated with antibody specific for *Salmonella* (Dynabeads™). Concentrated *Salmonella* cells are then infected with phage. After this any phage that have not infected a target cell are removed by washing, which also separates the cells from the magnetic beads. These infected, released cells are then incubated in broth to allow phage to complete their replication cycle and then the sample added to cells of a laboratory phage propagating strain (termed Signal Amplifying Cells or SACs).

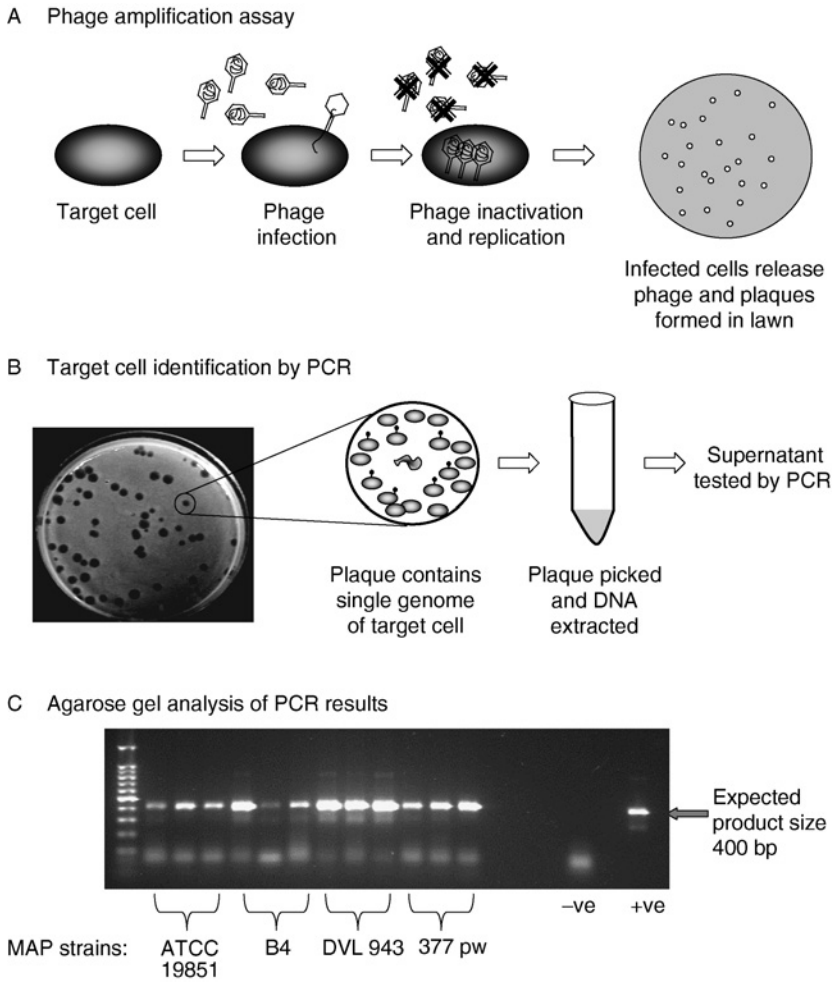

FIG. 2. Combined phage amplification-PCR assay: (Panel A) For the phage amplification assay, phage specific for the target cell are added to the test sample. Samples are incubated to allow phage to infect any target cells present and enter the eclipse phase when they begin to replicate inside the host cell. Virucide is then added to destroy any remaining exogenous cells. The virucide is then neutralized and infected cells mixed with a lawn of bacteria that will support phage replication. At the end of the infection cycle, the target cell is lysed and releases mature phage particles that can infect the host cells in the lawn and form a plaque. Each plaque represents a phage that was protected from the virucide by being inside a target host cell. (Panel B) Plaques formed after testing a sample for the presence of *Mycobacterium avium* subsp *paratuberculosis* (MAP) using the phage amplification assay. Each plaque contains the genomic DNA of the original target cell infected and many genomes of the bacteria used to form the lawn (in this case

If the optical density of this culture drops, this indicates that phage have been carried through the washing steps, protected within an infected cell, and this is a positive result for the presence of *Salmonella* cells in the original sample. If the optical density does not drop then no phage were carried through and hence the original samples did not contain *Salmonella* cells. As an alternative to optical density, LIVE/DEAD *Bac*light™ reagent could be used, with the fluorescent signal being proportional to the number of live SACs remaining. In each case, the detection limit achieved was less than 10^4 cells ml^{-1} in 4 to 5 hours.

IV. Phage in Hygiene-Monitoring Tests

In food processing environments, there is a need to monitor the effectiveness of cleaning regimes. Conventional culture methods do not provide results fast enough to allow remediation steps to be put in place if disinfection procedures have failed. Therefore rapid hygiene tests have been developed that measure the concentration of ATP released from bacterial cells remaining on a surface (Stanley, 1989). As dead cells rapidly deplete intracellular ATP levels, this provides an indication of the viable number of bacterial cells present. Many commercial companies produce such testing kits and in their most common format a bioluminescent assay is used where ATP concentration is determined by monitoring light produced by the enzyme firefly luciferase (Luc) in an ATP-driven reaction. Hence the more ATP present, the higher the light levels recorded. Test surfaces are swabbed (usually a 100 cm^2 area) and samples mixed with a releasing agent that breaks open any bacterial cells present. The substrate, luciferin, is then added to the sample and the light output from the luciferase enzyme recorded using small hand-held luminometers.

Individual bacterial cells from a given growth environment contain consistent levels of ATP (approximately 10^{-15} g per cell) and therefore the practical limit of detection for these assays is approximately 10^4 bacterial cells, and the assay does not provide any information about the nature of the cells detected (i.e., all bacteria are detected, not specifically pathogens). While useful for hygiene monitoring, it is

Mycobacterium smegmatis). Plaques are picked and DNA extracted from the agar for testing by PCR. (Panel C) Agarose gel results of PCR amplification of a 400 bp region of the MAP-specific insertion sequence IS900 using plaque DNA as PCR template. Three plaques arising from the detection of four different MAP strains were tested by PCR. As a negative control (−ve) DNA was extracted from a plaque arising from the detection of *M. smegmatis* by the same phage amplification assay. As a positive control (+ve) MAP genomic DNA was used as PCR template.

unlikely that pathogenic bacteria will be present on surfaces in this number and so a specific pathogen test for direct environmental sampling is probably of limited use. However, research has shown that phage can be used to selectively lyse pathogens in a mixed sample, adding specificity to this type of test, as shown by Sanders (1995) for the detection of *Listeria*. On its own, this would still not be suitable for direct testing of swab samples as, again, the numbers of cells present are too low to be detected by direct measurement of ATP. This limitation has been addressed by using a modification of the ATP assay where the signal is enhanced by the detection of released adenylate kinase. In essence, this enzyme generates ATP from ADP in a linear fashion and so the amount of light is proportional to the amount of enzyme released from bacterial cells. As for ATP, the amount of adenylate kinase per cell is relatively constant, and now fewer cells can be detected as the amount of ATP in the sample is increased by the enzyme activity above a threshold level (Corbitt *et al.*, 2000). Using phage as the lysing agent for this assay, the limit of detection can be reduced to less than 10^3 *E. coli* and *Salmonella* cells (Blasco *et al.*, 1998; Squirrel *et al.*, 2002; Wu *et al.*, 2001), which compares favorably with other rapid methods of bacterial enumeration, such as immunoassays.

V. Perspective

One reason for the lack of commercialization of phage-based tests is that they have to offer some additional benefit before being adopted in favor of rapid molecular detection methods. However, phage tests do provide an advantage over many molecular tests in that DNA-based detection methods cannot easily distinguish between live and dead, and cells containing cryptic genes which are not expressed may also be detected, giving rise to false-positive results. Molecular methods based on the detection of mRNA from genes being actively expressed (such as Reverse Transcriptase PCR) can differentiate between live and dead cells, but these methods are too expensive for routine food diagnostics and also the reagents are very sensitive to inhibition by contaminants from the food matrix, making the tests less robust and also more complex to perform. In phage-based tests, dead cells will not allow phage replication or allow expression of reporter genes so the cell detected must be viable. However false-negative results will always occur as phage-resistant mutants can usually be found in a population. Interestingly, it has been shown for Reporter Phage that the infection range of the host cells can be wider than the replication host range (i.e., strains

that will not propagate a phage will still support infection and expression of the reporter gene; Kuhn *et al.*, 2002).

When evaluating phage tests, results are often compared to culture methods, which are considered to be the ''Gold Standard.'' However, even using classical microbiological identification tests, atypical isolates can be recorded as false negatives. When comparing data sets, it is often found that phage tests detect cells in samples that are negative by culture, and vice versa. This suggests that both test methods have their limitations, but in reality both are equally effective at detecting a subpopulation of all of the cells present. Therefore the criticisms leveled at the phage-based assays do seem to be unfairly influenced by this type of comparison and this has hampered their development and acceptance as routine tests.

VI. Control of Bacteria in Food

The idea that phage could be used to specifically eradicate pathogenic bacteria arose very early after their discovery by Twort and D'Herrelle (Summers, 2005). Control of pathogens in the food chain can be addressed at all stages of production in the classic ''farm to fork'' approach. Bacteriophage have the potential to be utilized at a number of these stages; for example, zoonotic pathogen control could be exerted to prevent or reduce colonization of the live animal, or phage could be employed to decontaminate both carcass meat or for the disinfection of equipment contact surfaces. Phage intervention is also a potential strategy for the decontamination of other raw products, such as fresh fruit and vegetables, or in ready-to-eat produce where there would be no subsequent processing step to eliminate the pathogens. One of the criticisms of using phage in this way has been the potential for development of phage resistance that could soon result in the same problem of resistance encountered with the use of antibiotics and disinfectants. However, phage do produce some solutions to these problems themselves, through their natural abilities to evolve and overcome the resistance mechanisms of their hosts. Furthermore, the ubiquitous nature of phage in the same environments as their host means that their application in such situations is not adding a new component to the environment but merely changing the natural balance in favor of biocontrol. Such an approach is likely to be perceived as more acceptable to the consumer than chemical decontamination methods.

With these goals in mind, attention has therefore focused on finding lytic phage that could be used to control the major foodborne

pathogens and examined their use in a range of food or food production situations.

A. *Listeria monocytogenes*

Foodborne infection due to *Listeria monocytogenes* is often associated with either fresh or minimally processed foods, such as dairy products or salads, or with processed foods that are stored at low temperature. A psychrotroph, it not only forms part of the gut flora of many animals and, therefore, can be present in meat and milk, but also forms part of the saprotrophic population in soil and on leaf surfaces and so is a common environmental contaminant of fruit and vegetables. While not a very good competitor at higher temperatures, the organism has the advantage over many pathogens at low temperature in that it is able to continue growing under refrigeration conditions and can become endemic in cold storage facilities. Indeed, once established in biofilms, this organism can develop resistance to standard cleaning agents (Frank and Koffe, 1989). Hence, one of the first problems to which phages were applied was removal of *Listeria* biofilms. Roy *et al.* (1993) evaluated the effectiveness of a range of different phages to remove *Listeria* from stainless steel and polypropylene surfaces and were able to achieve approximately a 3 $\log_{10}$ drop in cell number using phage treatment alone. However it is never envisaged that phage would totally replace standard cleaning agents, rather they would be used as an adjunct. In this study, the phages used were also evaluated for their ability to withstand inactivation by a quaternary ammonium compound (QUATAL) used for cleaning, and it was found that they were not inactivated by concentrations up to 50 ppm. By using a combination of phage and 40 ppm QUATAL, a 5 $\log_{10}$ reduction in levels of surface attached *Listeria* was achieved. Hibma *et al.* (1997) also evaluated phage for the removal of *Listeria* from stainless steel. In this case, a phage was isolated that was specific for L-forms of *Listeria* where cell wall structure is either deficient or absent. Since successful phage infection is dependent on the expression of appropriate cell-surface structures, it is interesting that this group managed to isolate phage that could still efficiently infect *Listeria* cells with incomplete cell walls and shows that the ability of phage to infect host cells infection in the real world is more robust than we might predict.

As for other bacteria, phage have also been evaluated as a method to remove *Listeria* from foods. Leverentz *et al.* (2003, 2004) used a cocktail of phage that had been developed by the biotechnology company Intralytix (Baltimore, USA). This highlights a particular problem for

those wishing to use phage as an environmental biocontrol agent. In a case of human infection, it is likely that the bacterial population present in the patient is clonal, that is, it has arisen from the multiplication of one cell type that invaded the body. Hence cells can be cultured and tested for their phage-sensitivity before an appropriate phage is chosen for treatment. When trying to eradicate bacterial pathogens that are present in the environment, a much wider mixture of bacterial cell types are likely to be present and so cocktails of phage with as broad a coverage as possible need to be developed. This approach also has the advantage that it is unlikely that the cells present will be able to accumulate mutations to make them resistant to all of the different phage present, hence reducing the likelihood that phage resistant populations of bacteria will develop after exposure to phage treatment.

Leverentz *et al.* (2003) initially evaluated the phage cocktail for its ability to control *Listeria* on fresh-cut fruit (apples and melon) in combination with the bacteriocin nisin. As found when trying to detect bacterial pathogens using phage (Section III.B), differences in the food composition affected the efficiency of the phage treatment. In this case, although eradication of pathogens was achieved, the residual phage population rapidly declined on sliced apple surfaces so that no prolonged protection could be achieved. However, for both sliced melon and apples the phage were shown to work synergistically with the bacteriocin to achieve a greater overall reduction in cell number. A second study focused on the treatment of sliced melon and found that effective pathogen control could be achieved for up to 7 days storage at 10°C by treatment with phage alone, although, it was noted that for the treatment to be most effective it had to be applied soon after the fruit was cut (Leverentz *et al.*, 2004). This suggests that the phage need to attack the bacteria before they have a chance to be internalized into the plant tissue. In 2002, Intralytix were granted regulatory approval from the US Environmental Protection Agency (EPA) to trial the LMP 102 cocktail to prevent bacterial contamination of food and food processing plants. The EPA permit allowed Intralytix to undertake efficacy studies in actual plant operations as a prelude to commercial production.

Dykes and Moorhead (2002) also investigated the use of a combination of listeriaphage and nisin to reduce levels of *Listeria*. In this case, however, they used only a single phage isolated from human sewage but tested its ability to reduce levels of *Listeria* isolated from both a meat product and from a case of clinical listeriosis. While the same synergistic effect reported in the study of fruit was seen when cells in broth culture were treated, no reduction in cell number beyond what was achieved with nisin alone was seen when ground beef was treated

and stored at 4°C. The contrasting results achieved here highlights again the difficulty when developing phage-based methods for use in real foods as the food matrix itself is highly variable in composition and also the storage conditions and competitive microflora present will also influence the growth and survival of the target organisms. In reality, there will have to be extensive development work for each food type before phage can be used as an effective treatment.

B. *Escherichia coli* O157:H7

With the emergence of new pathogens, such as *Escherichia coli* O157:H7, there has been an interest in isolating phage that can rapidly be used for biocontrol in foods. Several groups have described phage that are specific to the O157:H7 serotype (Kudva *et al.*, 1999; Morita *et al.*, 2002; Tanji *et al.*, 2004). Perhaps not surprisingly, LPS structure was seen to be important for effective phage infection (Kudva *et al.*, 1999) and lysis rates were lower when cells were incubated below the threshold for growth and were, therefore, not actively growing. Sharma *et al.* (2005) used the phage described by Kudva *et al.* (1999) to remove cells attached to a surface in a biofilm. While they too had success in lysing planktonic cells or surface-attached cells, it was found that once cells had become established in complex biofilms they were protected from phage infection. This may have been due to the production of extracellular polysaccharides associated with biofilm development, or due to a change in the expression of surface structures required for phage infection.

O'Flynn *et al.* (2004) showed that three different phage could be used to reduce cell numbers in broth-grown cultures with a 5 $\log_{10}$ reduction achieved when cells were grown at 37°C. When low cell numbers were inoculated onto beef surfaces (10^3 cfu g^{-1}) held at 37°C, total eradication of cells was achieved when a cocktail of phage was applied in seven out of nine samples. In the remaining two samples, remaining counts were less than 10 cfu g^{-1}. The low cell number chosen was representative of the numbers of bacteria likely to be found on contaminated meat, and given that a 5 $\log_{10}$ reduction in cell number was achieved in broth cultures using the same phage cocktail, it would be expected that the bacteria would have been removed from all samples. This again illustrates the role of the food matrix in establishing effective methodologies, as modeling from liquid culture data is clearly inadequate. Another interesting feature of this study was an investigation of the occurrence of phage-resistant mutants after phage treatment. The frequency of phage resistance in the population was found to be

approximately 1 in 10^6 cfu. Given that a 1000-fold lower numbers than this are expected to be on the meat surface, the development of resistance is unlikely to be a major factor if phage are applied as a biocontrol agent. It was also interesting that the phage-resistance phenotype was unstable and cells had a coccoid morphology and grew more slowly. This suggests that resistant mutants are likely to be disadvantaged and would not easily become established in a population.

C. *Salmonella*

Salmonella typhimurium was one of the pathogens on which the early work using phage to control animal infection was centered (Sulakvelidze and Barrow, 2005). However in terms of control in food and food production, work has focused on two specific serovars of particular epidemiological significance: *S.* Enteritidis and *S. typhimurium* DT104.

Preharvest feeding trials with phage cocktails were carried out in poultry by Sklar and Joerger (2001) to control *S. enteritidis* colonization of the cecum. While average cecal counts of the inoculated *Salmonella* were 0.3–1.3 times lower than untreated infected birds, the ceca still contained between 10^5 and 10^7 cfu g^{-1}. Development of phage resistance to some of the phage was demonstrated but was not the sole factor allowing *Salmonella* survival. More recently, Fiorentin *et al.* (2005) have shown that oral treatment of birds artificially infected with *S.* Enteritidis PT4 with high numbers of bacteriophage (10^{11} pfu) reduced the concentration of *Salmonella* in ceca of broilers after 5 days by 3.5 $\log_{10}$ per gram. These lower levels persisted for up to 25 days after treatment but again the organism was not eradicated from the birds. However both studies show that the use of phage treatment would reduce numbers of organisms entering the poultry production line.

Modi *et al.* (2001) incorporated *Salmonella* phage SJ2 with the starter culture in production of cheddar cheese to examine the effects on the survival of *Salmonella.* This work was prompted by incidents of salmonellosis involving cheddar cheese as the food vehicle demonstrating the survival of *Salmonella* through the fermentation process. They used a strain of *S.* Enteritidis that had been associated with one of these outbreaks to spike the milk for cheese production. Both raw and pasteurized milk cheeses were made and the *Salmonella* was introduced postpasteurization at a level of 10^4 cfu ml^{-1} of milk and monitored through the production process to the final product. This work showed that when phages were incorporated, *Salmonella* did not survive in the pasteurized cheeses after 89 days, whereas they were

present at around 50 cfu ml^{-1} in raw milk cheeses. However, in contrast, all the cheeses without phage showed levels of 10^3 cfu g^{-1} after 99 days. Given that naturally contaminated product would have *Salmonella* at a much lower level, this demonstrates the potential of phage as an effective control for *Salmonella* in cheddar cheese production.

Leverentz *et al.* (2001) also looked at the control of *S. enteritidis* on fresh-cut apple and honeydew melon slices. They used a cocktail of four lytic phage, specific for *S. enteritidis*, to apply to the surfaces of the fruit. As was seen with *Listeria* studies, phage titers declined rapidly over 24 hours on the apples at all temperatures of storage (5–20°C) but much less rapidly on the melon; pH was considered to be the most likely inactivating factor as the pH of the apples at 4.2 was sufficient to experimentally inactivate the phage preparation. In line with this there was a reduction in levels of *Salmonella* on the melon but not on the apple. This may, therefore, be a limitation of such applications unless more pH-tolerant phage are used.

The wild-type strain and a variant (LP, large plaque) of the generic *Salmonella* phage Felix O1 have also been used to examine the potential for control of *S. typhimurium* DT104 (Whichard *et al.,* 2003). This strain is of particular concern due to its increased virulence and multi-antibiotic resistant nature. These authors examined control of the organism on chicken frankfurters and showed that both phage were able to suppress the levels of *Salmonella* by around 10^2 cfu g^{-1} when a high inoculum (300 cfu g^{-1}) and high-abuse temperature (22°C for 24 hours) were used. However, a much higher multiplicity of infection was needed than in broth to be completely effective. Again, studies need to be done using more realistic contamination levels and storage conditions, but the potential for the approach was demonstrated.

D. *Campylobacter*

Whereas biosecurity measures have gone a long way to reduce carriage rates of *Salmonella* in poultry flock, attempts to control the prevalence of *Campylobacter* in chickens have not been as successful. It appears that this organism is a natural commensal of the avian gut and, therefore, it is acquired very easily from a variety of environmental sources and quickly colonizes young birds. Hence recent work in phage biocontrol has focused on the eradication of *Campylobacter* from meat samples. One issue that must be addressed when devising a new food-processing step is that new substances cannot easily be added to foods without the need for extensive testing and regulatory approval. When initiating a programe that would potentially lead to treatment of birds

with phage, it was important to establish whether phage could naturally be found associated with poultry meat. This was answered by Atterbury *et al.* (2003) who showed that fresh meat stored at 4°C often has *Campylobacter* phage associated with it, with organic products being more likely to have phage than standard or economy products. Interestingly, freezing lead to a loss of phage recovery that suggests that physical methods could be used after phage treatment to inactivate them. In the same year, Goode *et al.* (2003) showed that they could apply *Campylobacter* phage to artificially contaminated chicken skin and achieve up to a 95% reduction in *Campylobacter jejuni* counts.

However, the ultimate aim would be to reduce levels in the bird prior to slaughter so that cross contamination during processing is reduced and levels remaining on the meat at the end of production are lowered. Atterbury *et al.* (2005) showed that a negative correlation existed between the presence of phage and levels of *Campylobacter* in cecal contents, indicating that some degree of biocontrol is occurring in natural populations and suggesting that artificial intervention should be successful. Two studies have described the treatment of birds with phage and reported reduction in levels of *Campylobacter* in cacal content (Carrillo *et al.*, 2005; Wagenaar *et al.*, 2005). In the Carrillo study, birds were experimentally infected and treated at 25 days using an antacid suspension to protect phage from acid denaturation. Results were very variable, with between 0.5 and 5 $\log_{10}$ reductions in *Campylobacter* numbers being recorded. Phage-resistant colonies were recovered but, like the *E. coli* O157 observations, these were physiologically compromised in that they rapidly reverted to a phage-sensitive phenotype upon laboratory culture. Wagenaar *et al.* (2005) investigated the use of phage both as a prophylactic treatment to prevent *Campylobacter* colonization and also as a therapeutic to treat birds that had been previously infected. When used therapeutically, *Campylobacter* numbers in the ceca declined by 3 $\log_{10}$ but eventually stabilized at levels only 1 $\log_{10}$ lower than the control group. When used prophylactically, colonization was delayed but eventually levels of *Campylobacter* similar to those in the therapeutic trial were achieved. Taken together, these studies suggest that a natural dynamic exists in the phage-host relationship that does not result in total eradication of the host. This is entirely to be expected of a system that has evolved to allow the survival of both host and virus, and indicates that phage treatment of birds is probably best confined to treatment immediately prior to slaughter to reduce levels of bacteria present but cannot be used as a biosecurity measure to keep *Campylobacter* out of broiler flocks.

E. Control of Food Spoilage Organisms

In contrast to pathogens, relatively little work has addressed the use of phage to control food spoilage. This may in part be because spoilage is a population dynamic; if one element of the population is specifically eliminated then another will become predominant. However, in some situations particular groups do predominate and replacement with a slower growing flora will allow a significant extension of shelf life.

Greer (1986) examined the use of phage for the control of *Pseudomonas* spoilage of fresh beef. Rib-eye steaks were inoculated with 10^6–10^7 cfu *Pseudomonas* and then between 0 and 10^8 pfu applied. Spoilage was evaluated after 4 days at 7°C under typical retail sale conditions. The work demonstrated that the phage could multiply on the steaks after infecting host cells and, at the highest concentrations used, could significantly affect both the growth of the bacterium and the appearance of the steaks in terms of surface decoloration. Similar results were found when phages were used to control the spoilage of pork adipose tissue by *Brochothrix thermosphacta* (Greer and Dilts, 2002). Interestingly in the latter studies, although spoilage (as measured by off-odor production) was controlled over 8-day storage under refrigerated conditions (compared with 4 days in the absence of phage), the *Brochothrix* counts were significantly reduced over the first 2 days and then growth recovered being almost at the same level as untreated controls at 10-day storage. A significant proportion (20–65% depending on trial) of the population of the organisms recovered at that time were then phage resistant but here no changes in their cell physiology were reported.

VII. Concluding Remarks

At the current time, all the uses of phage for biocontrol reported are at the experimental stage. Development to commercial application of all of these ideas will be influenced by the same factors as those that have limited the development of phage-based detection systems—specifically the product will have to provide an advantage over existing technologies before sufficient confidence will exist to attract the investment required. As the use of chemicals becomes more restricted, perhaps a niche will appear for surface sanitation methods. However as each phage treatment will target only one organism, it is likely that these will be specific applications where one organism causes a particular problem. The use of phage to reduce the entry of pathogens from animal

carcasses into the food chain does seem more promising. Generally there is one specific pathogen associated with a meat that the industry wishes to target and existing chemical decontamination methods are more limited, and likely to become more limited in the future. Two specters are generally raised as a criticism of phage-intervention methods. First, that pathogens are not totally eliminated. However existing methods are only expected to achieve a reduction in microbial load to lower the risk of transmission. If phage treatment is simply seen as a new adjunct to current food production processes to further reduce pathogen levels it can be seen as a useful contribution to the production of safe food, however, it is not a magic bullet that will eliminate the problem by itself.

The second concern raised is the emergence of phage resistance. If again we consider phage treatment as part of the food production line, and knowing that that the initial drop in pathogen levels directly after treatment is often the largest, it would suggest that treatment of animals immediately prior to slaughter would be the best strategy to employ. At this point, animals are being removed from the food production sites and, therefore, surviving resistant organisms are less likely to be released into the environment. Although not exhaustive, initial studies have also reported that phage-resistant organisms recovered from treated animals are often compromised by these mutations. In terms of phage evolution, this is not an altogether surprising finding since, over time, the phage which persist are those that propagate with best efficiency. If phage receptors were easily lost by a host cell, a rapid expansion of the phage-resistant population would be expected. Therefore, phage that target features of the bacterial cell, which is an advantage to the survival of that cell in the environment, are those that tend to persist since the cell can only lose the receptor at cost to themselves. As studies of bacterial phage-host ecology continue, it is likely that many more examples of phage-resistant mutants being less fit than their phage-sensitive parents will emerge. Hence if phage-resistance does become prevalent, it is probable that these organisms will be less likely to cause disease and therefore may actually add benefit to the use of phage treatments.

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Gastrointestinal Microflora: Probiotics

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I. Introduction

The human bacterial gut flora has been the subject of extensive investigation for several years. Its complexity, more than 400 commensal species, inaccessibility, and fastidious growth requirements constitute analysis an extremely painstaking task. The human gut, site of numerous diseases acute and chronic, also plays an important role in health promotion through the activities of the resident microflora. Probiotics have long been recognized as health-promoting agents. Advances in molecular methodologies and conduction of well-designed studies performed in the last two decades illustrate the potential of probiotics against a multitude of conditions.

II. The Human Gastrointestinal Microflora

The human adult colon is the most complex bacterial ecosystem in the human body, harboring more than 400 different culturable bacterial species belonging to more than 50 different genera (Finegold *et al.*, 1983; Gibson and Roberfroid, 1995). Due to gastric acid in the stomach

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 59 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(06)59007-0

and rapid washout rates in the small intestine the colon is the main site of bacterial colonization. The average bacterial content of the human colon is 10^{13} cells, while viable counts are typically in the region of 10^{12} cells g^{-1} of colonic contents (Gibson *et al.*, 1998). The composition of this complex and diverse ecosystem varies greatly according to the local anatomical characteristics and conditions such as pH, transit time of contents, and oxygen availability. Other factors affecting gut flora composition include diet, drugs, antibiotic therapy, and disease.

The study of gut microflora has relied almost exclusively upon the quantitative culture of fecal microorganisms. The picture of stool microflora obtained through culture techniques was far from accurate and the species enumerated accounted for only up to 50% to 80% of the total flora, with bacteroides and bifidobacteria being perceived as numerically predominant (Suau *et al.*, 1999; Tannock, 2001). The molecular view of the intestinal ecosystem is quite different from that obtained by microbial culture. The majority of studies based upon 16S rRNA analyses have confirmed that bacteroides is the predominant group in human adult feces accounting for up to 37% of the total bacterial flora. Different species of the genus *Eubacterium* account for up to 33%, while *Clostridium* and its relative genera can account for up to 60% of the total fecal bacteria (Harmsen *et al.*, 2002; Rochet *et al.*, 2001; Sghir *et al.*, 1999, 2000; Suau *et al.*, 1999). Bifidobacteria can rarely account for up to 15% of the total adult fecal flora and lactobacilli for about 1% (Table I).

The primary role of colonic flora is to salvage energy from dietary material that has not been digested in the upper gastrointestinal tract, through the process of fermentation. Different groups of the bacterial flora collaborate to degrade organic matter. It is postulated that about 7% to 8% of the total daily energy requirements of the host are derived from colonic bacterial fermentation (Gibson *et al.*, 1998).

Certain indigenous colonic bacteria are thought to be beneficial for host health, namely bifidobacteria and lactobacilli. Bifidobacteria have been linked with the good health of breast-fed infants and studies report a protective effect against infant gastroenteritis (Beerens *et al.*, 1980; Stark and Lee, 1982). Other species are benign, such as certain eubacteria, methanogens, and saccharolytic species of bacteroides and clostridia. Amongst the health-promoting actions of the colonic microflora is colonization resistance from external pathogens and prevention of overgrowth of internal harmful bacteria, facilitation of digestion, production of short-chain fatty acids (SCFA), trophic effect on the colonic mucosa anticarcinogenic activity, and stimulation of the immune system of the host. There are also those that are considered

TABLE I

THE ADULT COLONIC FLORA COMPOSITION AS DETERMINED BY BACTERIAL CULTURE AND MOLECULAR METHODS TARGETING 16S rRNA

Genus		Percent of total anaerobes				
		Culture methods			Molecular methods	
Bacteroides	G− rods	30	56	37	28	10.7
Bifidobacterium	G+ rods	11	4	1	4.8	2.4
Eubacterium	G+ rods	26	14	16	10.8	12.6
Fusobacterium	G− rods	8	0.1		NA	NA
Ruminococcus	G+ rods	4	9		10	NA
Clostidium	G+ rods	2	2		22.7	19.6
Lactobacillus	G+ rods	2	1	1	0.01	0.16
Streptococcus	G+ cocci	2	6	NA	NA	NA
Enterobacteriaceae	G− rods	NA	NA	1	0.2	0.04
Peptostreptococcus	G+ cocci	9	4	NA	NA	NA
Others	NA	3	1	NA	9.4	4.1
		Moore and Holdeman, 1974	Finegold *et al.*, 1983	Sghir *et al.*, 2000	Harmsen *et al.*, 2002	S. Kolida, unpublished 2005

G+: Gram positive; G−: Gram negative; NA: nonapplicable; species were not enumerated for.

detrimental for human health. Among the most important colonic pathogens are *Clostridium difficile, Clostridium perfringens,* sulfate-reducing bacteria such as *Desulfovibrio* spp., proteolytic members of *Bacteroides*, and pathogenic enterobacteria. Colonic pathogens have been associated with the production of toxins, carcinogens, and pre-carcinogens, toxic gases, such as hydrogen sulphide, and can contribute toward the manifestation putrefaction. It is evident that the human colon harbors a uniquely complex and metabolically active ecosystem that plays an invaluable role in the host well-being. The different populations of the colonic microbiota exist in a dynamic but delicate balance, and the complexity of the flora is necessary for the fulfillment of its metabolic function. If this balance is disturbed and pathogenic bacteria increase, then chronic or acute disease can occur.

With the advent of medicine during the last century, the life expectancy of the human population has increased by an average of 20 years during the past 50 years. Today there are approximately 600 million people over the age of 60. This total is expected to double by 2025 (WHO, 2003). This increase will pose a very heavy burden on national healthcare systems. At older age the beneficial components of the human microflora decrease, while potential pathogens such as clostridia increase (Mitsuoka, 1992). This would lead to a higher incidence of gastrointestinal diseases such as cancer, inflammatory bowel disease (IBD), antibiotic associated diarrhea, irritable bowel syndrome, and constipation. It is imperative to explore alternative approaches not only to protect from, but also treat gastrointestinal diseases. Years of antibiotic overuse has increased bacterial resistance to current pharmacological treatments. As such, alternative courses of action against disease have to be sought out. Evidence from historic use of fermented milk products as health-promoting agents present an intriguing approach: the probiotic approach to health management.

III. Probiotic History

The protective nature of certain microorganisms, in particular lactic acid bacteria contained in fermented foods and drinks has a long history. Humans have been consuming live bacterial cultures for centuries in the form of fermented milk without any knowledge of the active ingredients or how they work. Among the first reported probiotic intakes was the ingestion of soured milk by nomads over 2000 years ago. In the start of the last century, Metchnikoff attributed the good health and longevity of Bulgarian peasants to the ingestion of kefir, a

fermented milk product (Metchnikoff, 1907). He isolated what he named as Bulgarian bacillus, which is currently known as *Lactobacillus delbrueckii* ssp Bulgaricus. During the First World War, the physician Nissle isolated a strain of *Escherichia coli* (*E. coli* Nissle 1917) from the only healthy soldier in a group suffering from infectious diarrhea. He suggested that this strain was responsible for protection from diarrhea due to its ability to suppress other pathogenic bacteria (Nissle, 1925). Such empirical observations provided the foundations upon which the probiotic concept was based and developed.

IV. Definition of Probiotics

The term probiotic is derived from the two Greek words: ''προ'' and ''βιος'' and literally means ''for life.'' In 1991 Fuller defined probiotics as:

> Live microbial food supplements that beneficially affect the host by improving the intestinal microbial balance.
>
> —(Fuller, 1991)

It is difficult to identify with certainty the first time the term probiotic was used, but it is believed that one of the earliest mentions was by Vergin in 1954 when he suggested that the intestinal microbial balance may be upset following antibiotic use, and that it could be restored by a diet of probiotics, including fermented foods (Vergin, 1954). The term was reintroduced in 1965 by Lilly and Stillwell who defined probiotics as ''substances produced by microorganisms which promote the growth of other microorganisms,'' the antonym of antibiotics (Lilly and Stillwell, 1965). The definition was improved about 10 years later by Parker (1974), who defined probiotics as: ''Organisms and substances which contribute to intestinal microbial balance'' which is closer to the designation proposed by Fuller in 1991. In the past 14 years, several attempts were made to improve on Fuller's definition of a probiotic to a more general form that includes beneficial effects of probiotic microorganisms in other sites apart from the colon, such as the urinary tract, or address the issue of adequate bacteria levels:

> Probiotic is a preparation or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host.
>
> —(Havenaar and Huis In't Veld, 1992)

> A probiotic is a live microbial culture or cultured dairy product which beneficially influences the health and nutrition of the host.
>
> —(Salminen, 1996)

> Probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit to the host.
>
> —(FAO/WHO, 2002)

Although the above definitions address valid points, they have not managed to effectively improve Fuller's definition. Probiotics (bacteria, foods) are a very heterogeneous group and as such it is very difficult to summarize their qualities in a short definition. Mentions on host health imply freedom from disease, and this is not always the case with probiotic supplementation, while probiotic foods are not just limited to dairy products. The most widely accepted definitions in literature are still Fuller's (Fuller, 1991) and the one proposed by the World Health Organization (FAO/WHO, 2002).

V. Microorganisms Currently Used as Probiotics

Commercially available probiotics commonly contain strains of the *Bifidobacterium, Lactobacillus*, and *Streptococcus* genera as well as yeasts of the *Saccharomyces* genus. The majority of commercially available probiotic formulations contain lactobacilli as opposed to bifidobacteria as the former are more aerotolerant and thus more stable in food products (Crittenden and Playne, 1996). In Europe, the most common probiotic delivery formats are fermented dairy products, such as milk drinks and yoghurts (Table II), as well as lyophilized preparations.

The majority of probiotic foods are not associated with specific health claims and general statements on their efficacy are preferred during marketing such as:

'helps support your body's natural defences'

'feel good bacteria for healthy digestion'

'wellness from the inside'

Any health claim associated with a probiotic food product is strictly regulated by the Natural Health Product Directorate (NHPD) in Canada, the Food and Drug Administration (FDA) in the United States, and the European Food Safety Authority (EFSA) in the European Union (EU). All three authorities call for strong scientific proof on any health claim associated with a probiotic food (Sanders *et al.*, 2004). Contrary to what is proposed in Canada and the United States, EU regulations prohibit

TABLE II

PROBIOTIC FOOD PRODUCTS MAINLY AVAILABLE IN EUROPE. PROBIOTIC STRAINS ARE PRESENTED AS DESCRIBED ON PRODUCT PACKAGING

Trade name	Probiotic culture	Producer
Actimel	*L. casei* Immunitas	Danone
Activia	*B. lactis*	Danone
Gefilus	*L. rhamnosus* GG	Valio
Enjoy	*L. acidophilus, Bifidobacterium*	Valio
Yakult	*L. casei* Shirota	Yakult
LC1	*L. johnsonii* LA-1	Nestlé
Biopot	*L. acidophilus, B. longum, S. thermophilus*	Onken
Vifit Vitamel	*L. rhamnosus* GG	Campina
Vitallity	*L. acidophilus, Bifidobacterium*	Müller
Vita Fresh	Lactic cultures, *B. bifidus*	Mevgal SA
Ageladitsa plus	*B. bifidus*	Fage AE
YogActive	*L. acidophilus*	Belgo & Bellas
ProViva	*L. plantarum* 299V	Skånemerjerier
Caserio Bio	*L. reuteri* Protectis	Kraft Jacobs Suchard Iberia
Cultura	*L. casei* F19	Arla Foods
O'soy	*L. acidophilus, L. casei, L. reuteri, Bifidobacterium*	Stonyfield Farm
Life top™ straw yogurt drink	*L. reuteri*	Orchard Maid

any claims referring to prevention, treatment, or cure of a human disease for a food. Only disease risk reduction claims are to be allowed.

VI. Selection Criteria for Probiotic Bacteria

For a microorganism to be characterized ''probiotic,'' a number of criteria have to be met (Dunne *et al.*, 2001; Tannock, 1998):

1. Human origin. Although certain commercially available probiotics are not of human origin, it is believed that if a probiotic is isolated from the human gastrointestinal tract it is safer for human consumption and may be more effective in colonizing the large intestine.

2. GRAS (generally regarded as safe) status. GRAS status is granted by the FDA to food/food components that have been proven to be safe for human consumption through scientific procedures or through experience based on common use in food, based on a substantial history of consumption by a significant number of consumers. Bifidobacteria and lactobacilli have a long history of safe consumption without any harmful effects on human health.
3. Probiotics must be capable of being prepared on a large scale and in a viable manner. It is also very important to be viable and active in the specific delivery vehicle.
4. Probiotics have to be resistant to gastric acidity and bile acid toxicity. The low gastric pH is one of the primary host defense mechanisms against ingested microorganisms, including probiotics.
5. Adherence to human intestinal cells and intestinal mucins, which will improve persistence and multiplication in the colon and promote competitive exclusion of potential pathogens from mucosal surfaces.
6. Production of antimicrobial substances against gut pathogens for the restoration of a healthy microflora composition.
7. Safety in food and during clinical use even in immunocompromised individuals.
8. Efficacy and safety proven in randomized, double-blind, placebo-controlled human studies.

As our knowledge on probiotic efficacy and action progresses, the list of criteria grows longer to include factors, such as efficacy against *H. pylori* infection, antimutagenic and anticarcinogenic properties, antioxidative activity, immune system stimulation, to name but few. It is obvious that no single probiotic strain can fulfill all of the above-mentioned criteria. Understanding the mechanisms of target diseases should aid identification of the necessary probiotic selection criteria for a specific health application. Recent advances in encapsulation technologies allow for direct delivery of probiotic bacteria to target sites, as such gastric and bile acid resistance do not pose a limitation in certain cases. The necessity of viable culture use has been put forward as certain studies have reported health effects from heat-killed probiotics. Although such preparations can induce an immune response, they cannot be involved in competitive exclusion of potential pathogens, trophic effect on gut mucosa, and all other benefits associated with cells being metabolically active (Kaila *et al.*, 1995; Kirjavainen *et al.*, 2003; Pessi *et al.*, 1999).

Unlike pharmaceuticals and food additives, until recently, the quality criteria for probiotics were not well defined. The viability and purity

of probiotic preparations is of the utmost importance for both the functionality and the safety of probiotic products. Most of the clinical effects of probiotics have been reported for viable strains. However, recent reports in the United States and UK demonstrated the lack of adequate controls in this respect. In the United States, out of 30 probiotic formulae tested, 11 contained no viable bacteria (Weese, 2003). It appears that the bacterial contents of probiotic formulae are commonly misidentified or misspelled, while information on the viable number of cells is often inaccurate and misleading. Several products were also found to contain the potential pathogen *Enterococcus faecium* (Hamilton-Miller *et al.*, 1999; Temmerman *et al.*, 2001). In 2002, the WHO recognized the need for guidelines for the evaluation of probiotics and identified the minimum requirements for probiotic status (Fig. 1). Their recommendation was that the genus, species, and strain designation should be given on the product label and that the strain designation should not mislead the consumers as to strain functionality. In addition, the minimum viable numbers for each strain, the shelf life, and proper storage conditions of the product and the required dose to affect a health claim, along with any reported health claims, should all be available on the label (FAO/WHO, 2002).

Another important aspect of probiotic use is safety. Historically, lactobacilli have been used extensively in food processing, and bifidobacteria being the predominant species in the intestine of healthy, breast-fed infants, have also been considered to contribute to health. Recently however, cases of systemic infections with a possible correlation to common probiotic strains as *L. rhamnosus* and *L. paracasei* have been documented (Lee *et al.*, 2004; Mackay *et al.*, 1999; Rautio *et al.*, 1999; Schmidt *et al.*, 2001). All cases occurred in individuals with underlying medical conditions and a compromised immune system.

In vitro and *in vivo* animal studies provide very useful insight on probiotic efficacy and safety, while they are necessary in determining acid and bile tolerance. The only proof of probiotic health-promoting effects accepted by different regulatory bodies worldwide is substantiation of claims through human studies. As such we will focus on the evidence on probiotic efficacy obtained from human studies.

VII. Lactose Intolerance

The term lactose intolerance describes the inability to digest significant amounts of lactose. This inability results from a shortage of the enzyme lactase, which is normally produced by the cells that line the small intestine. Common symptoms include nausea, abdominal

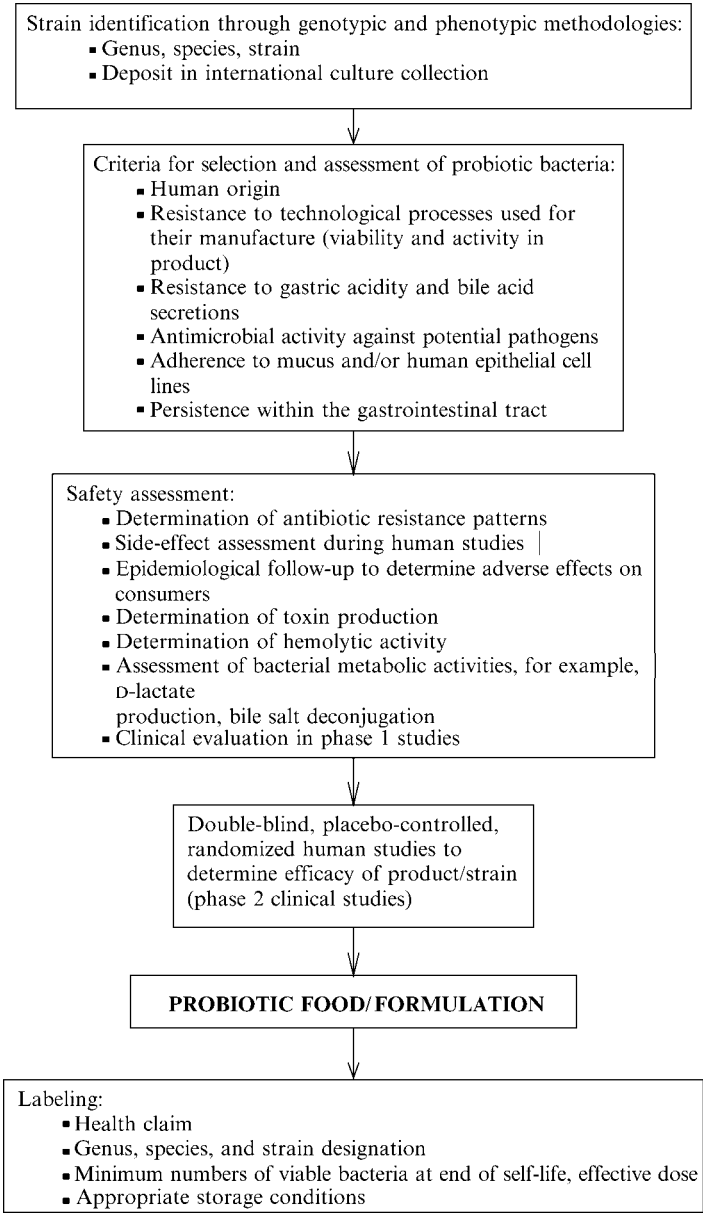

FIG. 1. WHO guidelines for the evaluation of probiotics for food use (modified from FAO/WHO, 2002).

cramps, bloating, gas, and diarrhea, which begin about 30 minutes to 2 hours after eating or drinking foods containing lactose. Lactose intolerance is a common disorder that may affect as many as 70% to 75% of the world population (Szilagyi, 2004).

Alleviation of lactose intolerance by probiotics has been one of the first probiotic effects to be demonstrated (Marteau *et al.*, 1990). ''Yoghurt bacteria'' (*S. thermophilus* and *L. delbrueckii* subspecies bulgaricus), are nowadays recognized as probiotics (Guarner *et al.*, 2005) and usually considered as the most efficient to improve lactose digestion and alleviate symptoms of lactose intolerance. As they are not resistant to bile secretions, they are easily lysed in the small intestine, which allows for their enzymes to be released (Marteau *et al.*, 1997).

A systematic review (Levri *et al.*, 2005) assessing the efficacy of oral probiotics in randomized, controlled trials published from 1966 to 2002, excluding *S. thermophilus* and *L. delbrueckii* subspecies bulgaricus, in adults with lactose intolerance concluded, however, that only specific strains at specific concentrations are effective, but that in general, probiotic supplementation did not alleviate the symptoms of lactose intolerance in adults. This is possibly because these strains have not initially been selected for this purpose and are too resistant to bile secretions. Recently a study testing the multiprobiotic product VSL#3 also failed to improve lactose maldigestion (Yesovitch *et al.*, 2004).

The case of lactose maldigestion is a very good example to illustrate that although fulfillment of the aforementioned probiotic selection criteria is regarded as an indication of a potentially successful probiotic, characteristics should be selected considering the mechanism of the disorder targeted to ensure efficacy.

VIII. Atopic Disorders

During the last two decades a steep increase in the incidence of atopic diseases, such as eczema, asthma, allergic rhinitis, and cow's milk allergy has been observed. The intestinal microflora plays a central role in the immune system regulation in the intestinal mucosa associated lymphoid tissue (MALT), which, in turn, is very important for atopic sensitization. Important features of atopy, the excess formation of IgE to food antigens, for example, unhydrolyzed casein, and the development of T helper 2-skewed immune responsiveness, are targets of probiotic activity (Majamaa and Isolauri, 1997).

Increasing evidence is suggesting that ingestion of certain probiotic strains may be a very effective way to mediate antiallergenic effects and

alleviate symptoms of atopic diseases. It has been reported that certain probiotic bacteria are able to stimulate the production of Th1 cytokines (Hessle *et al.*, 1999; Miettinen *et al.*, 1998), transforming growth factor beta (Paganelli *et al.*, 2002), and gut IgA (Fukushima *et al.*, 1999; Kirjavainen *et al.*, 2003).

Since the first clinical trial that demonstrated efficacy of probiotics to modify allergic inflammation (Isolauri *et al.*, 2000), only a limited number of clinical studies have shown that probiotic ingestion could be beneficial. The best evidence is focused on food allergy and atopic dermatitis. Concerning other allergies, clinical evidence is sparse. One study carried out on pollen allergy failed to show any improvement of symptoms when *L. rhamnosus* GG (LGG) was administered (Helin *et al.*, 2002). So far, most of the studies have been carried out with lactobacilli and in particular with LGG (Isolauri *et al.*, 2000; Kalliomaki *et al.*, 2001; Viljanen *et al.*, 2005). This strain has been shown to down-regulate milk-induced inflammatory response in milk-hypersensitive subjects but has an immunostimulatory effect in healthy subjects (Pelto *et al.*, 1998). However, combining LGG with three other probiotics suppressed the beneficial effects seen with LGG alone, perhaps due to an interference of immunostimulating effects between the strains (Viljanen *et al.*, 2005). Prenatal administration of LGG to pregnant women 1 month prior to delivery and postnatal administration for 6 months reduced incidence of atopic eczema in at-risk children during the first 2 years of life (Kalliomaki *et al.*, 2001). This preventive effect was observed after a 4-year follow-up of the same group (Kalliomaki *et al.*, 2003). An outline of studies on probiotics against atopic disorders is presented in Table III.

Although evidence supporting probiotic efficacy against atopic diseases is quite convincing, further studies investigating the mechanisms of disease pathogenesis are required.

IX. Treatment and Prevention of Diarrhea

A. Antibiotic Associated Diarrhea

Antibiotic associated diarrhea (ADD) is defined as an acute inflammation of the intestinal mucosa caused by the administration of broad-spectrum antibiotics. It occurs to approximately 20% of patients receiving antibiotic treatment. Antibiotic treatment disturbs the balance between commensal colonic bacteria populations. Overgrowth of *C. difficile* is the most common etiology of ADD, being responsible for more than 40% of reported ADD cases (Clabots *et al.*, 1992).

TABLE III

CLINICAL STUDIES WITH PROBIOTIC TESTED TO ALLEVIATE THE SYMPTOMS OF ATOPIC DISEASES

Study design	Patients	Probiotic	Dose	Conclusions	References
Randomized, double blind, placebo controlled	230 children under 1-year with cow-milk allergy	LGG or mixture of four probiotics (LGG, *L. rhamnosus* LC 705, Bb99, *Propionibacterium* JS)	5×10^9/day 4 weeks	LGG alleviated symptoms of food allergy but only in IgE sensitized volunteers Probiotic combination with LGG suppressed the effects seen with LGG alone	Viljanen *et al.*, 2005
Randomized, double blind, placebo controlled	53 children between 6 and 18 months with atopic dermatitis	*L. fermentum* PCC	3×10^9 twice daily for 8 weeks	Improved extent and severity of atopic dermatitis	Weston *et al.*, 2005
Double blind, placebo-controlled, cross-over study	Infants (mean age 4.6 months) with atopic dermatitis	*L. rhamnosus* 19070–2 and *L. reuteri* DSM 122 460		Strains beneficial in the management of atopic dermatitis. Effect more pronounced in patients with positive skin prick test response and increased IgE levels.	Rosenfeldt *et al.*, 2003
Double blind, randomized, placebo controlled	Prenatal in mothers and postnatal for 6 months in their infants.	LGG		LGG was effective in prevention of early atopic disease in children at high risk	Kalliomaki *et al.*, 2001

(*continued*)

Table III (*Continued*)

Study design	Patients	Probiotic	Dose	Conclusions	References
Randomized, placebo controlled	27 children with atopic eczema mean age 4.6 months	LGG and BB12 in extensively hydrolyzed whey formulas	3×10^{8} 10 ^{9}/g 2 months	Efficacy of probiotics to modify allergic inflammation. Significant improvement in skin condition occurred in patients given probiotic-supplemented formulas	Isolauri *et al.*, 2000
Double blind, randomized, placebo controlled	38 young adults and teenagers allergic to birch wood	LGG	5×10^{9}/day 6 months (pollen season)	The treatment did not alleviate the symptoms during the birch pollen season	Helin *et al.*, 2002
Placebo controlled	15 children with atopic dermatitis	*B. breve* M-16V		Significant improvement of allergic symptoms	Hattori *et al.*, 2003

Probiotic consumption has been shown to decrease AAD incidence. *S. boulardii* and *L. rhamnosus* GG both proved effective against AAD in several human studies with a large number of subjects (Table IV). *S. boulardii*, a patented yeast preparation, has already been used in many countries for several years to prevent and cure AAD. It is able to inhibit the growth of several pathogens both *in vivo* and *in vitro*, survives the gastrointestinal tract, and most importantly stays unaffected by antibiotic therapy (Boddy *et al.*, 1991). However, in one study in elderly patients on antibiotics, it failed to decrease the appearance of *C. difficile* (Lewis *et al.*, 1998). LGG was more successful in reducing the rate of diarrhea occurrence in children taking antibiotics (Arvola *et al.*, 1999; Vanderhoof *et al.*, 1999) than in adults where no effects were observed (Thomas *et al.*, 2001).

B. Infectious Diarrhea

Infectious diarrhea is the most common cause of diarrhea worldwide and is the leading cause of death in childhood, especially in the developing world. It can be viral, parasitic, or bacterial and can account for up to 3 million deaths each year among children less than 5 years of age (Casburn-Jones and Farthing, 2004). Rotavirus is the most common cause of severe diarrheal disease and dehydration in infants and children worldwide and a major cause of death.

A large number of well-designed intervention studies against rotavirus diarrhea has demonstrated probiotic efficacy in infants (Isolauri *et al.*, 1991; Kaila *et al.*, 1992; Majamaa *et al.*, 1995) with *L. rhamnosus* GG showing the more consistent effect in reducing the duration of diarrhea when compared to placebo (Szajewska *et al.*, 2001). Specific rotavirus IgA was shown to be produced when certain probiotics were ingested (Kaila *et al.*, 1992). Viable probiotics were more efficient in inducing these responses than inactivated probiotics (Kaila *et al.*, 1995). In a recent review by Allen *et al.* (2004) where 33 placebo-controlled trials were analyzed, it was concluded that probiotics appear to be a useful adjunct to rehydratation therapy in treating acute, infectious diarrhea both in adults and children but that more research is needed to define the use of particular probiotics regimens in specific patient groups. Studies also suggest that probiotic efficacy is more prominent if supplementation occurs at early disease stages.

C. Traveler's Diarrhea

Bacteria, such as enterotoxigenic *E. coli*, enteroaggregative *E. coli, Campylobacter, Salmonella*, and *Shigella*, are common causes of

TABLE IV

CLINICAL STUDIES WITH PROBIOTICS USED IN ANTIBIOTIC-ASSOCIATED DIARRHEA

Study design	Patients	Probiotic	Dose	Conclusions	References
Randomized, placebo controlled	267 patients	LGG	2×10^{10}	LGG did not reduce the rate of occurrence of diarrhea in this sample of adult patients taking antibiotics initially administered in the hospital setting.	Thomas *et al.*, 2001
Placebo controlled	188	LGG	10^{10}	LGG reduces the incidence of antibiotic-associated diarrhea in children treated with oral antibiotics for common childhood infections.	Vanderhoof *et al.*, 1999
Randomized, placebo controlled	Children with acute respiratory infection	LGG	2×10^{10} twice daily	LGG is effective in the prevention of diarrhea in children receiving antimicrobial treatment to respiratory infections.	Arvola *et al.*, 1999
Double blind, randomized, placebo controlled	246 children with otitis and/or respiratory tract infections	*S. boulardii*	250 mg twice daily	Lower prevalence of diarrhea in children	Kotowska *et al.*, 2005

Randomized, placebo controlled	69 patients	*S. boulardii*	113 g twice daily	No evidence that the concomitant use of S. boulardii with antibiotics alters patients' bowel habits or prevents the appearance of *C. difficile* toxin in the stool.	Lewis *et al.*, 1998
Double blind, randomized, placebo controlled	124 adults with *C. difficile* disease (CDD)	*S. boulardii*	1 g/day for 4 weeks	The combination of standard antibiotics and *S boulardii* was shown to be an effective and safe therapy for these patients with recurrent CDD; no benefit of *S boulardii* was demonstrated for those with an initial episode of CDD.	McFarland, 1994
Double blind, randomized, placebo controlled	180 hospitalized patients	*S. boulardii*		Significant difference in the incidence of diarrhea (22% in the placebo group against 9% in the group receiving the probiotic)	Surawicz *et al.*, 1989

traveler's diarrhea, which affects millions of people traveling to developing countries each year (Yates, 2005).

Evidence on beneficial preventive effect of traveler's diarrhea by probiotics is inconsistent. A few studies have reported a prophylactic effect with LGG (Hilton *et al.*, 1997; Oksanen *et al.*, 1990) and *S. boulardii* (Kollaritsch *et al.*, 1993). Other studies reported negative results with lactobacilli (Katelaris *et al.*, 1995; Pozoolano *et al.*, 1978).

Probiotic efficacy against traveler's diarrhea is the least substantiated health claim of all three diarrhea categories, the reason being that the aim of probiotic supplementation is not focused on treatment, but mainly prevention of diarrhea occurrence.

X. Ulcerative Colitis and Pouchitis

Ulcerative colitis is a chronic relapsing inflammatory disease of the large bowel, which has an unknown etiology. It is a primary, often recurrent, and occasionally persistent mucosal inflammatory condition, which mainly affects the rectum but can subsequently spread to a variable length of the colon. It is characterized by diffuse inflammation of the wall of the large bowel, predominantly confined to the mucosa and superficial submucosa, and is usually associated with diarrhea and rectal bleeding.

Incidence rates are highest in Scandinavia, the United Kingdom, and North America but low in eastern and southern Europe (Maybery *et al.*, 1991). The chronic nature of the disease and the need for constant medication poses a major financial burden on health systems. To date, there is no cure for the disease and treatment has so far been limited to maintenance of remission. Currently, drug therapy is mainly based on the administration of antiinflammatory and immunomodulating drugs, nutritional support, and in severe cases, surgical resection.

Observations from germfree rodents in which intestinal inflammation cannot be induced indicate that bacteria are necessary for the pathogenesis of chronic intestinal inflammation (Sartor, 1997; Veltkamp *et al.*, 2001). It has been suggested that IBD is at least partially due to a breakdown of tolerance to the normal commensal colonic flora (Macpherson *et al.*, 1996) or disturbed colonic flora (Pathmakanthan *et al.*, 1999). Several studies have investigated the fecal flora of ulcerative colitis patients trying to identify the causative agent or an abnormal bacterial load. Among the possible candidates suggested were *E. coli* (Darfuille-Michaud *et al.*, 1998), sulphate-reducing bacteria (Florin *et al.*, 1990; Gibson *et al.*, 1991; Levine *et al.*, 1996; Pitcher *et al.*, 2000), mycobacteria (Hermon-Taylor *et al.*, 2000; El-Zaatari *et al.*,

1999), pseudomonas (Chiba *et al.*, 2001), and helicobacter species (Fox *et al.*, 1999; Kullberg *et al.*, 1998).

Several lactobacilli have been tested in various mouse and rat models of ulcerative colitis, including *L. reuteri, L. plantarum, L. rhamnosus* R2LC, and genetically modified *L. lactis* (to produce IL-10) reporting improvement in disease symptoms, colonic barrier function, and restoration of a healthy microflora (Fabia *et al.*, 1993; Holma *et al.*, 2001; Madsen *et al.*, 1999; Mao *et al.*, 1996).

Human studies thus far have mainly evaluated two probiotic products: nonpathogenic *E. coli* Nissle 1917 and VSL#3. VSL#3 is a very high-concentration (450 billion live bacteria per sachet) blend of eight different bacterial strains: *L. acidophilus, L. plantarum, L. casei, L. delbrukii* ssp. *bulgaricus, B. breve, B. infantis*, and *S. salivarius*.

Studies investigating the efficacy of *E. coli* Nissle 1917, have focused on demonstrating that probiotic treatment is equally effective with what is considered by some to be the golden standard of ulcerative colitis maintenance treatment, mesalazine. In two recent double dummy, double blind studies with ulcerative colitis patients taking oral mesalazine or capsules containing a nonpathogenic strain of *E. coli* Nissle 1917, the probiotic treatment had an equivalent effect to mesalazine in maintaining remission of ulcerative colitis (Kruis *et al.*, 1997, 2004). The same effects were observed by a second research group in 116 ulcerative colitis patients with active disease in a similar design study (Rembacken, 1999).

The efficacy of VSL#3 as a maintenance treatment in 20 ulcerative colitis patients allergic or intolerant to sulfasalazine or mesalazine was initially shown by Venturi *et al.* (1999). As many as 15 patients achieved remission and fecal levels of lactobacilli, bifidobacteria, and *S. salivarius* subspecies *thermophilus* significantly increased. Results were recently confirmed by a different group reporting that out of 34 mild to moderate ulcerative colitis patients, nonresponding to conventional treatment regimes, 77% achieved remission upon VSL#3 intake. The gut mucosa tissue samples endoscopically obtained from three of the patients achieving remission were analyzed using PCR/DGGE, *S. salivarious* subspecies *thermophilus*, and *B. infantis* were detected. None of the three patients examined had populations of these species prior to the study (Bibiloni *et al.*, 2005). This indicates that bacteria contained in this formula do reach the colon and certain strains manage to adhere to the colonic mucosa successfully. The above-mentioned probiotic preparations were evaluated in well-controlled human studies and findings were replicated in more than one research centers using well-defined probiotic products.

Other less-substantiated claims from pilot studies also exhibited encouraging results. Ishikawa *et al.* (2003), conducted a randomized clinical on the use of a bifidobacteria fermented milk (BFM) administered with the maintenance treatment. Disease relapse was observed only in 3 of the 11 volunteers on BFM as opposed to 9 out of 10 of the control groups. Guslandi *et al.* (2003) assessed the efficacy of *S. boulardii* supplementation in a pilot trial of ulcerative colitis patients in mild to modrate relapse. Volunteers received 250 mg of *S. boulardii* for 4 weeks during mesalazine maintenance treatment. Of the 24 patients participating in the study, 17 attained clinical and endoscopic remission.

Pouchitis is the most frequent, long-term complication, following the procedure of pouch surgery, for the treatment of ulcerative colitis, and is characterized by a nonspecific inflammation of the ileal reservoir. Several studies exhibited the potential benefits of probiotic administration in pouchitis treatment and/or prevention and the probiotic efficacy in this condition is the best established. As with ulcerative colitis treatment, VSL#3 has proven quite beneficial as opposed to single probiotic strain approaches. Gionchetti *et al.* (2000) evaluated the efficacy of VSL#3 in a double-blind, placebo-controlled trial of 40 patients suffering from chronic pouchitis in clinical and endoscopic remission. Nearly 100% of the placebo group relapsed as opposed to only 15% of the VSL#3-treated group. In a later study, Gionchetti *et al.* (2003) investigated the efficacy of VSL#3 administration in preventing the onset of pouchitis during the first year following ileal–pouch anastomosis in a double-blind, placebo-controlled trial. A significantly lower number of volunteers on the VSL#3 treatment developed acute pouchitis (10%) as compared to 40% of the patients receiving the placebo treatment. More recently, Mimura *et al.* (2004) further confirmed the above observations in a two-centre study of 36 pouchitis patients. Patients received either VSL#3 (300 billion bacteria/g) at 6 g once daily, or placebo. Only 6% of the placebo group remained in disease remission as opposed to 85% of the VSL#3 group. Thus, treatment with VSL#3 appears efficient in preventing the onset as well as relapses of the condition.

Single bacterial strain treatments have proven less effective. Kuisma *et al.* (2003) investigated the effect of *L. rhamnosus* GG supplementation in a double-blind, placebo-controlled trial in 20 patients with active pouchitis. Probiotic supplementation changed the pouch flora by increasing the fraction of total fecal lactobacilli to total fecal anaerobes. However, no differences in disease activity were observed between the placebo and the active treatment group.

Further studies are required to establish the efficacy of probiotics in ulcerative colitis and pouchitis treatment and prevention. It is possible that the beneficial effect may be species specific, or that a synergy between different bacterial species is required for efficacy in IBD management. This may explain the lack of efficiency of the single strain probiotic formulations tested thus far. The main problem in the management of IBD is that both the etiology and the mechanism of the disease are largely unknown. Although there is logic behind the probiotic approach in its management, selection of probiotic strains is a process of trial and error without a specific target. The issues of dose and treatment duration have not been addressed yet. Different doses of the same probiotic/formulation have not been evaluated to determine the minimum effective dose. Nevertheless, the fact that probiotic treatment has proven effective as maintenance treatment for IBD and that to a certain extent the process appears to be strain specific in the case of pouchitis may shed light in the mechanisms of disease pathogenesis.

XI. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders affecting 3% to 15% of the general population (Cremonini and Talley, 2005). It is associated with the manifestation of diarrhea or constipation or both alternating, accompanied by abdominal bloating and pain. The cause is not as yet known and colonic biopsies from IBS patients appear to be normal. Although it is suggested that IBS is a psychosomatic disorder, the colonic microflora has been implicated in its pathogenesis (Balsari *et al.*, 1982; Bradley *et al.*, 1987; Madden *et al.*, 2001).

Studies on probiotic efficacy in the treatment of IBS have yielded conflicting results so far. *L. casei* did not have any effect on disease symptoms (O'Sullivan and O'Morain, 2000) but oral supplementation with *L. plantarum* proved effective in reducing flatulence and abdominal pain in two double-blind, placebo-controlled studies (Niedzielin *et al.*, 2001; Nobaeck *et al.*, 2000). The effect was not observed though in a further similar design study with *L. plantarum* (Sen *et al.*, 2002). Brigidi *et al.* (2001), administered VSL#3 to 10 IBS patients and observed an improvement of patient clinical picture and significant increase in lactobacilli, bifidobacteria, and no significant difference in bacteroides, enterococci, and *C. perfringens* levels. The same authors reported, in a later randomized, controlled trial testing the same probiotic formulation in 25 IBS patients with diarrhea-predominant disease, that no significant effect was observed. A more recent study on VSL#3

efficacy in improving symptoms in diarrhea-predominant IBS reported a marginally significant decrease in abdominal bloating, while other symptoms such as abdominal pain, gas production, and urgency remained unaffected (Kim *et al.*, 2003).

Evidence of probiotic efficacy on IBS symptom relief is sparse and conflicting and further studies are required. Our understanding of the disease is very poor and fundamental studies on factors mediating its pathogenesis are required. It is likely that efficacy of treatment is going to depend not only on probiotic used, but also the type, diarrhea, constipation, or alternation between the two, of the disease.

XII. Probiotics Against Urogenital Tract Infections

More than 300 million women worldwide currently suffer from nonsexually transmitted urogenital infections such as urinary tract infection (UTI), bacterial vaginosis, and yeast vaginitis. Although hygiene standards have improved during the past 40 years, incidence of urogenital infections has tripled (Reid and Bruce, 2003).

The female urogenital tract is populated by a *Lactobacillus*-dominated microflora, which similarly to the large intestine, acts as a natural barrier against infection from pottentially pathogenic microorganisms. Studies so far indicate that the numerically predominant *Lactobacillus* species in the urogenital tract are *L. crispatum* and *L. iners*, while *L. acidophilus, L. gasseri*, and *L. delbrukii* exist in lower numbers (Antonio *et al.,* 1999; Burton *et al.,* 2003).

Bacterial vaginosis is caused by an overgrowth of aerobic microorganisms combined with a decrease in commensal *Lactobacillus* levels. UTIs are caused by Gram-negative bacteria such as *E. coli* and *Enterococcus faecalis*. For yeast vaginitis *Candida albicans* is considered as the main infectious agent. The common denominator in all the above conditions is that the main source of pathogens infecting the urogenital tract is the human colon. It is thought that pathogens ascend from the rectum to the vagina and that this might be how the urogenital flora is initially acquired. The main factors influencing urogenital flora are the local pH, hormone levels, menstruation, urine, semen, and spermicide use. Lactobacilli show optimum growth at low pH and any factors that increase vaginal pH, such as semen, will affect the indigenous flora composition (Reid and Bruce, 1995). Spermicides, such as nonoxynol-9, have been shown to kill vaginal lactobacilli (McGroarty *et al.*, 1992). In contrast, it has been shown that in the presence of estrogen, lactobacilli increase their adhesion to epithelial cells (Chan *et al.*, 1984).

As changes in *Lactobacillus* populations are considered to be involved in the pathogenesis of urogenital infections and antibiotics seem to contribute toward this, alternative treatments such as probiotics have been considered. The rationale behind probiotic use against urogenital infections is based on three possible mechanisms of action:

1. Increased ascension of probiotic and/or commensal lactobacilli from the rectum to the vagina.
2. Improvement of colonic flora composition so that potential pathogens do not infect the urogenital tract.
3. Enhancement of intestinal musocal immunity, which also improves vaginal immunity.

Indeed there is growing evidence that certain *Lactobacillus* strains can protect the host against urogenital tract infections. Different properties of lactobacilli that may contribute to their ability to protect against uropathogens have been identified:

1. Hydrogen peroxide production
2. Lactic acid production
3. Biosurfactant production (Velraeds *et al.*, 1996)
4. Resistance to spermicides

Currently, the most studied and effective *Lactobacillus* strains for the treatment of urogenital infections are *L. rhamnosus* GR-1 (Reid *et al.*, 1987, 1995). Although it can colonize the human vagina, it does not produce hydrogen peroxide but it is resistant to spermicides. The mechanisms, which constitute different strains effective while others are not, appear to vary, and different combinations of the above-mentioned traits are thought to give them the advantage.

Studies so far have exhibited that there is potential for lactobacilli to be used as therapeutic agents in urogenital infections, but questions related to the site and manner of probiotic delivery, dose and treatment duration are yet to be answered.

XIII. *Helicobacter pylori* Infection

Helicobacter pylori (*H. pylori*) is the causative agent of chronic gastritis and idiopathic peptic ulcer disease. *H. pylori* infection has been associated with increased incidence of gastric cancer of which 60–80% is attributable to *H. pylori* (Asghar and Parsonnet, 2001). Standard treatment for *H. pylori* infection is 1-week therapy combining two antibiotics together with acid suppression (triple therapy). Although triple therapy is quite effective, fully eradicating *H. pylori* from the

stomach is a difficult task mainly due to inadequate prescriptions, bacterial resistance, and poor patient compliance. Antibiotic administration causes gastrointestinal tract side effects, which further compromise patient compliance. Although the main site of *H. pylori* infection is the stomach, probiotics have recently been considered as possible treatment.

Two main hypothesis/approaches are currently considered:

1. Probiotics against *H. pylori*. Probiotics are used as the sole treatment against *H. pylori* to kill or suppress stomach levels.
2. Probiotics to alleviate triple therapy side effects and improve *H. pylori* eradication rates. Probiotics are used in conjunction to triple therapy to alleviate gastrointestinal side effects, such as diarrhea, caused by antibiotic administration.

The majority of studies administered probiotics in either yoghurts or fermented milks. *L. gasseri* yoghurt has been reported to suppress *H. pylori* levels and reduce gastric mucosal inflammation (Sakamoto *et al.*, 2001). A combination of *L. acidophilus* and *B. lactis* Bb12 (AB-yoghurt) significantly decreased urease activity after 6 weeks of ingestion as controlled to placebo (Wang *et al.*, 2004). Wendakoon *et al.* (2002), though, failed to observe a change in urea breath test upon ingestion of probiotic yoghurt containing *L. acidophilus* and *L. casei*.

When AB-yogurt containing bifidobacteria was combined with triple therapy, *H. pylori* eradication was higher in the probiotic group than in the triple therapy group alone while fecal *Bifidobacterium* levels were also restored in the probiotic group (Sheu *et al.*, 2002). Freeze-dried *Lactobacillus rhamnosus* GG combined with triple therapy minimized gastrointestinal side effects, such as diarrhea, nausea, and taste disturbance, caused by triple therapy administration (Armuzzi *et al.*, 2001). In a more recent study combining a probiotic milk based drink containing a mix of *L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve* Bb99, and *Propionibacterium freudenreichii* ssp. *shermanii* JS or placebo, to triple therapy failed to see a significant improvement in gastrointestinal side effects, but when the total symptom score change was examined it was significantly lower in the probiotic group (Myllyluoma *et al.*, 2005). Sykora *et al.* (2005) observed an enhanced therapeutic benefit on *H. pylori* eradication in children when triple therapy was combined with *L. casei* DN-114–001 fermented milk.

Although very interesting observations on probiotic efficacy against *H. pylori* infection and treatment side effects have been made, research is still in its infancy. The mechanisms mediating probiotic efficacy have not been investigated. Clearly the fact that most probiotic strains

are chosen to be resistant to gastric secretions may facilitate their efficacy, but as gastric transit is relatively fast it is not certain if they can target *H. pylori* in the stomach. Not all studies exhibited a beneficial effect and this poses questions on strain specificity and effective dose in particular, as in the majority of studies thus far, probiotics were delivered in yoghurts and fermented milks. The observations of the above studies will need to be confirmed and the identity of the probiotic microorganisms contained in the formulations used should be carefully defined using the latest methodologies.

XIV. Future Perspectives

In the last few decades, the need to find replacement therapeutic approaches to overcome side effects associated with the current pharmacological treatments, and the need for new antimicrobials due to the overuse of antibiotics has propelled forward research on probiotics against a multitude of disorders of varying severity. The findings are promising in most of the earlier reviewed conditions but the major obstacle that we have yet to overcome is not only the lack of knowledge with regards to the mechanisms of action of probiotics themselves but also of the target diseases. In the majority of cases, probiotic selection is an informed guess. Understanding the pathogenesis of complex diseases, such as IBD, will facilitate successful selection of efficient probiotics. Other aspects to be considered are the definition of a minimum effective dose for a specific probiotic strain to exert a specific health effect. Thus far this has only been defined empirically. Different conditions may require different probiotic dose to be administered while treatment duration and delivery vehicle may also differ. Target disease parameters should always be carefully considered as issues on probiotic safety on immunocompromised patients have been posed. In multistrain probiotic products, individual strain activities should be first defined and compared with the multistrain formulation to justify the reason for using them in combination. Although probiotic strain combinations may result in a synergistic or additive effect on activity, it may be also result to antagonism between different strains and, as such, be inferior to single-strain formulas.

Despite the challenges that have to be faced, new probiotic applications continue to emerge against diseases, such as colon cancer and cardiovascular disease, which are the two leading causes of death in Westernized countries.

In conclusion, further studies are required not only to elucidate the mechanisms of probiotic action in different conditions but also on the etiology of the diseases to be targeted while large-scale,

double-blind, placebo-controlled studies are essential to substantiate claims on probiotic efficacy.

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The Role of Helen Purdy Beale in the Early Development of Plant Serology and Virology

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I. Introduction

Helen Purdy Beale (1893–1976), a pioneer in plant virology, used *Tobacco mosaic virus* (TMV) to establish tools for plant virus serology that are now standard practice in research and diagnosis. In 1929, Beale reported that serum collected from rabbits after serial injections of TMV-infected sap had antigenic properties not associated with healthy tobacco sap. This was important because it showed that TMV was a discrete pathogen and TMV-specific antiserum could be used as a reagent to discriminate between TMV and other viruses. The TMV-specific serum was also used to track TMV as it was purified from plants as a crystalline material and to visualize the virus by electron microscopy. Beale's role in the discovery of the nature of TMV was profoundly affected by her career at the Boyce Thompson Institute for Plant Research and the influence of (and interactions with) some of the leading virologists of the day including Louis O. Kunkel, Francis O. Holmes, and Wendell M. Stanley. Beale developed the quantitative techniques of immunology and serology for the plant virus community in the early 20th century, yet it was not until the 1960s that her tools came into general use for diagnostics and experimentation.

When Helen Purdy Beale arrived at the Boyce Thompson Institute for Plant Research in 1924, plant virology was in its infancy. Only two decades earlier, Martinus W. Beijerinck, working with TMV, had established that viruses were submicroscopic and filterable pathogenic agents—features that distinguished them from bacteria and fungi.

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 59 0065-2164/06 $35.00

DOI: 10.1016/S0065-2164(06)59008-2

Yet the true nature of the virus remained an enigma for the next 30 years (Scholthof, 2004; Scholthof *et al.*, 1999).

During these intervening years, speculation abounded about the nature of plant viruses. Some scientists continued to ascribe virus diseases to bacteria or protozoa; others came up with new explanations altogether. In 1902, Albert F. Woods, a plant physiologist with the United States Department of Agriculture (USDA), proposed that a zymogen (or enzyme) was the causal agent of ''the true nature of the mosaic disease of tobacco'' (Woods, 1902). His colleague Harry A. Allard disagreed. From his experiments, Allard reasoned that the causal agent was not an enzyme since ''a tiny drop of virus diluted to 1 part in 10,000 can readily produce the mosaic disease'' (Allard, 1915). He found the enzyme theory ''not only needless but illogical'' because it required an assumption ''that the immeasurably small quantity of oxidizing enzymes [sic] carried by this drop [must be] sufficient to increase the normal oxidase content already present in the plant to the extent of a permanent pathological reaction resulting in the mosaic disease'' (Allard, 1915). Allard concluded the mosaic disease of tobacco was an ''ultramicroscopic parasite of some kind'' (Allard, 1916).

For the next decade, plant pathologists performed thermal and dilution endpoints and determined that mosaic diseases (viruses) could be transmitted by grafting, infected tubers or seed, rub inoculation, and aphids. Most of the work was descriptive such as monitoring symptoms and recording cellular abnormalities by light microscopy. Although the enzyme theory had been discounted by Allard (1916), prevailing scientific opinions suggested protozoa, colloids, globulins, or viroplasms were the causal agents of virus diseases (Creager, 2002; McKinney, 1972; Rivers, 1928; Stanley, 1941a).

During this era, Helen Purdy Beale also played an important role in exploring the nature of viruses. Her contribution was to bring serological techniques to plant virology. She was the first to show that virus-specific antibodies were powerful reagents for the identification and characterization of TMV, a crucial step in understanding the nature of the virus. She showed that it was possible to neutralize the infectivity of TMV with specific antiserum, that TMV in various host plants could be detected by antiserum derived from TMV-infected tobacco, and that other viruses were not detected by TMV-specific serum. She used statistical analyses and bioassay techniques in conjunction with antiserum to begin to chemically define the nature of viruses.

Helen Alice Purdy was born at Croton-on-Hudson, New York on September 19, 1893. Little is known about her early years, but she likely had an interest in botany and chemistry by the time she went

off to college. She took college preparatory studies at the Ossining School in Ossining, New York before attending Barnard College in New York City in 1914. In 1918, she completed an A.B. in botany. Following Barnard College, she began Ph.D. studies in plant pathology at Cornell University. Cornell had recently created a Department of Plant Pathology and already was a leader in training young practitioners in the field (Campbell *et al.*, 1999). During 1918–1919, Beale worked as a research assistant for Herbert Hice Whetzel, who established the Department and served as its head. For reasons that are unknown, Beale postponed completion of her Ph.D. work, but undoubtedly, her experience at Cornell gave her a picture of both fundamental and applied plant pathology.

For the next several years, Beale held a series of overlapping jobs and fellowships. She was an instructor of biology at Vassar (1919–1920) and then spent a year on an American–Scandinavian Foundation fellowship at the University of Copenhagen (Purdy and Walbum, 1922). She gained further expertise in bacteriology and serology at the New York City Department of Health research lab under the direction of William H. Park (Hammond, 1999). In 1921, she passed the New York State examination to become an accredited laboratory assistant in bacteriology. From 1922–1923, Beale was an assistant to William A. Murrill, a mycologist at the New York Botanical Garden, followed by a year at the Hawaiian Sugar Planters' Association in Honolulu as a Bishop Museum Fellow. She returned to New York in 1924 and applied to an advertised position for a plant pathologist at the Boyce Thompson Institute. A major turning point in her career occurred when she was hired by Louis O. Kunkel to work with plant viruses (Crocker, 1948; Maramorosch, 1996).

II. Critical Years at the Boyce Thompson Institute

The Boyce Thompson Institute for Plant Research, Incorporated, formally opened in September 1924, in Yonkers, New York. Colonel William Boyce Thompson, who had made a sizable fortune in mining, founded, built, and endowed the new Institute on his Yonkers estate. Thompson was motivated both by his passion for plants, particularly asters, and by a vision that science could serve as an instrument for human progress. Aware of the work conducted on human diseases at the Rockefeller Institute for Medical Research, Thompson desired to embrace public health through the novel approach of creating his own Institute where research would be focused on the health of the nation's food supply. Thompson believed that growing healthier food crops

through a much fuller understanding of plant physiology and pathology was just as essential to public health goals as sanitation, hygiene, and the prevention of human disease (Crocker, 1948). Thompson later was quoted as stating some years before founding the Institute, ''When I have enough money, I am going to build a laboratory to study some of the fundamental things. I want to do something to get at the bottom of the phenomena of life processes and I think a good place to study them would be in the realm of plants. Any principles concerning the nature of life that you can establish for plants will help you to understand man, in health and in disease. So, by helping men to study plants, I may perhaps be able to contribute something to the future of mankind'' (Hagedorn, 1935).

The Boyce Thompson Institute opened to much fanfare, heralded as the most advanced facility for plant science research in the world. Newspapers greeted the Institute as ''A Temple of Plant Science'' and ''The Workshop of a New Burbank.'' President Coolidge sent a message that was read at the dedication expressing to Thompson his ''appreciation for the pioneer work you are doing through the Boyce Thompson Institute for Plant Research, Inc. The scientific mastering of the problem of plant life will strengthen that base of all material welfare-agriculture'' (Robins, 1924). Coolidge added that this ''is the new pioneering in the American Advance'' (Robins, 1924).

Thompson had spared no expense in planning the new facility. He sought the advice of the nation's leading plant scientists, including John M. Coulter of the University of Chicago, Lewis R. Jones from the University of Wisconsin, and Herbert H. Whetzel from Cornell. William Crocker, associate professor of Plant Physiology at the University of Chicago, became the first director in 1921, charged with planning and organization. Crocker traveled across the United States and Europe, visiting leading botanical laboratories and experiment stations for ideas as well as using the opportunity to purchase books for an extensive library. When the Institute officially opened its doors in 1924, the more unusual features for the first time in plant biology laboratories were the accurately controlled temperature rooms and constant condition light and dark rooms, modern photography facilities, sophisticated greenhouses equipped with sensitive environmental controls, and spectral glass that made it possible to test the effects of various portions of the sun's rays on plant growth (Coulter, 1926; Crocker, 1948; Zimmerman, 1929).

Among the first scientists at the Boyce Thompson Institute was Louis O. Kunkel, who actually had arrived at Yonkers in 1923 to work on yellows disease of plants, a particular problem on Thompson's

prized asters. Kunkel had gained recognition for his work the previous three years on leafhopper and aphid transmitted diseases for the Hawaiian Sugar Planters' Association. Just 4 months after his arrival in Hawaii, he had discovered the specificity of plant virus transmission by insects (Holmes, 1960; Peterson, 1998). At the Boyce Thompson Institute, Kunkel quickly assembled a group of scientists with diverse backgrounds to work on various aspects of yellows and virus disease problems. Beale was among this initial group.

Soon after she was hired at the Boyce Thompson Institute as a member of Kunkel's virology group, Beale began her quest for understanding the nature of the virus. One of the problems that interested her was the issue of whether or not bacteria were the causal agent of TMV. She followed the work of Peter K. Olitsky of the Rockefeller Institute, who claimed that TMV could be grown in a filtrate of healthy tobacco sap (Olitsky, 1925). Beale's own research demonstrated that Olitsky's claim was wide off the mark. In her experiments, she was ''unable to obtain any evidence that the active agent producing mosaic disease in tobacco and tomato plants multiplies outside the living plants'' (Purdy, 1926). Beale was not alone in her conclusions; her findings were in agreement with those of Maurice Mulvania, a graduate student at the University of Wisconsin working under the direction of James Johnson (Mulvania, 1925).

Beale's lack of ability to culture TMV *in vitro* may have led her to experiment with detached leaves. She developed a bioassay of TMV accumulation, using serial dilutions from detached tobacco leaves for 11 passages (Purdy, 1928c). Beale conclusively showed the development of the infection by staining epidermal leaf peels with 0.5% aqueous solution of iodine green. The ''host nuclei appeared turquoise blue while the intracellular bodies became pinkish,'' she reported (Purdy, 1928c). Francis O. Holmes, also of Kunkel's virology group at the Boyce Thompson Institute, had suggested using this technique, published by Bessie Goldstein, a graduate student in the Botany department at Columbia University under the direction of R. A. Harper (Goldstein, 1924, 1926). Goldstein was the first to report the presence of TMV-associated inclusions including virus aggregates (crystals) and x-bodies (Goldstein, 1924, 1926).

A third paper by Beale appears to have been in response to a practical problem—an infestation of garden slugs in the Institute's greenhouses. This brief report showed that slugs were not able to transmit TMV to host plants (Purdy, 1928b). Even in this instance, Beale set up well-organized experiments, repeated the data by transferring slugs from plant to plant, used both tobacco and tomato, and two species of slugs.

She found that when slugs were macerated after feeding on TMV-infected leaves and used for inoculum they ''readily produced infection'' (Purdy, 1928b).

While pursuing these early experiments on TMV, with the encouragement of Kunkel, Beale returned to Cornell for continued graduate studies in 1925. Whetzel had encouraged Beale to complete an MS degree, which would make her competitive for jobs at the USDA or an experiment station. Both institutions were major avenues for employment for budding plant pathologists, the USDA particularly for women (Campbell *et al.*, 1999; Rossiter, 1982). Problems developed, however, although their nature is unclear. After working ''very diligently for a year,'' Whetzel refused to sign her thesis. According to Karl Maramorosch, Beale was ''so upset and furious'' by the experience that ''she threw the pages into Whetzel's face, screaming 'You have shown the claws of the devil!''' Immediately she ''stormed out of his office, packed her belongings,'' and left Ithaca (Maramorosch, 1996).

Beale returned to Boyce Thompson where Kunkel again ''strongly encouraged her to continue studies toward a Ph.D. degree'' (Maramorosch, 1992). Around 1927, Beale started coursework at Columbia University. Kunkel provided Beale with a full-time position as an assistant plant pathologist while she was enrolled at Columbia in the Department of Bacteriology. Her coursework at Columbia put her in contact with Fredrick Parker Gay, who had joined the Department of Bacteriology within the College of Physicians and Surgeons in 1923. Prior to this appointment, Gay had established and chaired the Department of Bacteriology and Pathology at the University of California-Berkeley. Both Gay and William Park, of the New York City Department of Health, provided Beale with the exposure to cutting-edge bacteriology and serology techniques, including complement fixation. A course by Claus W. Jungeblut on the use of immunology for the diagnosis of human diseases triggered an idea for Beale to ''investigate the antigenic properties of tobacco mosaic virus. This opened the way for serological identification of TMV and other plant viruses,'' the subject of her Ph.D. dissertation (Maramorosch, 1996).

Beale had an idea that a substance in animal serum, an antibody, could be used to limn the nature of a plant virus (Purdy, 1929). Beale realized that the tools of serology could transform virology and plant pathology, in particular to tease out the biochemical features of a virus. In 1929 she completed her dissertation on ''Immunological Reactions with Tobacco Mosaic Virus'' and published the results in the *Journal of Experimental Medicine* (Purdy, 1929). It is a remarkable paper.

For her experiments, Beale prepared plant sap from healthy plants and TMV-infected plants. She found that antiserum generated from

TMV sap was different from healthy sap serum. Furthermore, when the healthy sap was repeatedly cross absorbed with TMV antiserum, interaction with normal sap was abolished. Using the cross-absorbed serum, which was now TMV specific, she obtained very clear and reproducible precipitation reactions (Fig. 1). This elegant (and simple) experiment was the crucial demonstration that TMV was a discrete substance that accumulated in tobacco mosaic diseased plants. Beale also showed that TMV-specific antiserum could be used as a reagent to determine if plants were infected with TMV (Fig. 1). This was an important piece of additional evidence that TMV was not a host-induced protein, but the agent of disease. Beale's research provided the definitive observations on the application of serology to study the properties of plant viruses (Purdy, 1928a, 1929).

In her work, Beale had also suggested that her findings ''may be of value for rapid and accurate determination of the relative concentration of virus extracts'' (Beale, 1933) in coordination with local lesion assays. Holmes, also at the Boyce Thompson Institute, had discovered that *Nicotiana glutinosa* leaves developed conspicuous local necrotic lesions (but not systemic infections) when inoculated with TMV-infected sap. His results, published in the *Botanical Gazette* in 1929, showed that local lesions could be used as a direct assay for TMV and to isolate pure cultures of the virus (Holmes, 1929; Scholthof, 2004; Shaw, 1999).

Beale found that *N. glutinosa* plants infected with TMV had much lower virus titers using the sap:serum precipitin assay than similar assays with TMV-infected *N. tabacum* plants (Fig. 1). In the discussion she merely reported this observation as due to the local lesions (i.e., a lower titer indicates less virus). It appears that Beale did not recognize that she had developed a powerful means to monitor the genetics of resistance and movement of TMV by comparative analysis using the two tobacco cultivars and her TMV strains. Holmes later crossed the *N* (necrotic) gene from *N. glutinosa* into economically important tobacco cultivars to develop resistance to TMV via a hypersensitive response that obviates systemic infections (Holmes, 1938; Scholthof, 2004). Today, this is described as a gene-for-gene interaction between the plant *N*-gene protein and the TMV 126-kDa protein, a component of the TMV RNA-dependent RNA polymerase (Scholthof, 2004; Soosaar *et al.*, 2005).

Following her Ph.D. in 1929, Beale was awarded a National Research Council Fellowship. While she continued at the Boyce Thompson Institute, Beale was appointed as a Research Associate Bacteriologist in the Department of Bacteriology at Columbia University. The Fellowship resulted in a second important paper, where she showed that specific agents in TMV antiserum interfere with infectivity and that

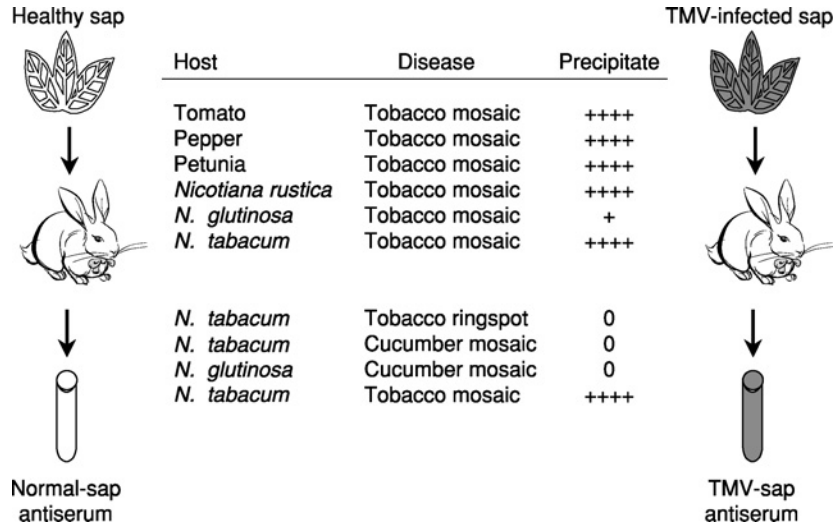


FIG. 1. Precipitin-absorption tests with sap serum from TMV-infected tobacco plants to determine if the filterable virus is a host protein or specific agent. The drawings represent healthy plants (white) and TMV-infected (grey) tobacco plants. To prepare the sap for injection in rabbits it was centrifuged ''at high speed for 15 minutes'' (Purdy, 1929) and the supernatant fluid was diluted in normal saline. The TMV antiserum was cross absorbed with healthy plant material prior to its use for the precipitin assays. From these experiments Beale observed that a heavy precipitate formed, indicated by increasing numbers of ''+'' signs, when virus-sap serum was mixed with sap tobacco mosaic diseased tobacco, tomato, pepper, or petunia plants. *Nicotiana tabacum* is the Turkish cultivar, a systemic host for TMV. No precipitate formed, indicated by a ''0,'' when adding normal sap of the same plants. She concluded that the ''antigenic property [i.e., TMV] is apparently not altered by multiplication in different hosts'' (Purdy, 1929). Beale showed that similar tests with unrelated viruses including *Tobacco ringspot virus* and *Cucumber mosaic virus* did not form a detectable precipitate (Beale, 1931). Beale also developed cross-absorption protocols to reduce nonspecific interactions, allowing for fine discrimination of such effects as the very low titer associated with TMV infections of *N. glutinosa*, a local lesion host identified by F. O. Holmes (Beale, 1931; Holmes, 1929).

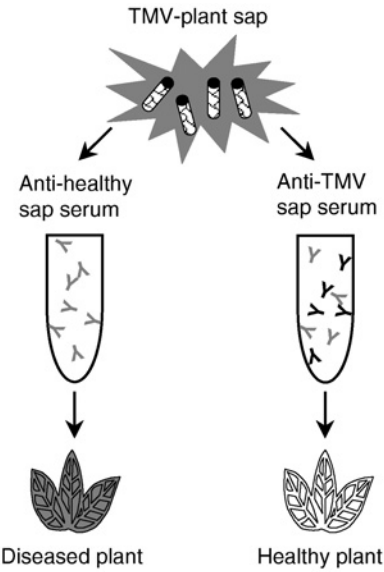
serological assays could be used to determine virus titer (Beale, 1931). She also developed a novel approach to directly assay TMV for virus: antigen specificity by modifying a medical bacteriology technique. As a student of F. P. Gay at Columbia University, she would have been familiar with Jules Bordet's bioassay, using antiserum for identification of bacterial species (Bordet, 1909). In the assay, if the antibody:antigen interaction is incomplete, it is possible to plate the mixture and count colonies. In contrast, if the antibody:antigen reaction is complete (fixed), then colonies will not be observed. With Bordet's assay, it

was possible to determine if there was a mixture of bacteria in the sample and to further characterize them by staining, morphology, and serology.

In Beale's variation, she assayed healthy and TMV-infected plants in a pair-wise fashion, using antiserum produced in rabbits to either healthy sap or TMV sap (Fig. 2). If the TMV antiserum:antigen interaction was complete then the virus would be neutralized and no infection would occur. If the interaction was incomplete (TMV sap:healthy antiserum), then the plants should become infected. This was in fact the case, and in such assays she found that an average of 88% of the plants inoculated with TMV sap:TMV antiserum remained healthy compared with 30% inoculated with TMV sap:healthy-sap antiserum, or TMV sap preincubated with normal serum (6% healthy), or saline (0% healthy). Beale's development of this plant virus-neutralization assay provided a biologically relevant means to demonstrate that antiserum had a specific inhibitory effect on the infectivity of TMV *in planta*.

The test was not absolutely conclusive, as shown by the control experiments, but Beale wrote that ''in respect to the power of inactivation of virus-sap exhibited by the antiserum to virus-sap, in comparison to that shown by antiserum to normal sap, the results are so significant as to require no comment'' (Beale, 1931). She was the first to show the utility of this assay for plant virology. And she again extended this work by showing that the TMV antiserum was specific to TMV-infected plants and did not react with *Tobacco ringspot virus* or *Cucumber mosaic virus* infections of tobacco. Thus, she showed that serum derived from healthy plants is nonspecific and had derived a method to prove that anti-TMV serum specifically identified TMV in infected host plants (Fig. 1).

With the two reports on using serology to study plant viruses (Beale, 1931; Purdy, 1929), Beale had established new and powerful techniques for plant virologists. But significant changes were underway at the Institute. In 1932, Kunkel moved to the Rockefeller Institute of Medical Research in Princeton, New Jersey as the director of the new division of plant pathology (Creager, 2002). Holmes joined him and soon thereafter, Wendell M. Stanley was hired to work on the biochemistry of plant viruses (Creager, 2002). Although Beale stayed on at the Boyce Thompson Institute, by 1932 its virology program had for all practical purposes been dissolved, in part because of a decision made during its founding to not duplicate work conducted elsewhere. Rapidly, the Rockefeller Institute took the lead in plant virology research (Creager, 2002), while work lagged behind at Boyce Thompson.



Serum used to inactivate TMV	# Plants inoculated	# Plants diseased	# Plants healthy	% Plants healthy
Anti-TMV serum	89	14	75	90
Antisap serum	20	14	6	30
Preimmune serum	10	8	2	20
Saline	26	26	0	0

FIG. 2. A bioassay to determine if TMV antiserum could reduce or abolish the infectivity of TMV sap. The healthy- and virus-sap antisera were prepared as in Fig. 1. Freshly extracted virus sap was added to antibodies (indicated by ''Y''-shapes) raised to either healthy sap or TMV-infected sap (Purdy, 1929). After incubation at room temperature for several hours or at 37°C for 1 hour, ''the contents were shaken thoroughly, poured into a dish and used for inoculation'' (Purdy, 1929) to *N. tabacum* cv. Turkish, a systemic host plant for TMV. Three leaves of each plant were inoculated and symptoms recorded for 4 weeks. Beale also prepared a battery of control treatments including plants with normal sap, normal serum from rabbits and guinea pigs, and saline solution. The results of this experiment show that the addition of healthy-sap serum had some inhibitory effects, as first reported by Mulvania (1926). Beale showed that the interaction of TMV with its homologous serum results in up to 90% healthy plants (i.e., 10% infection) compared to 100% infection (grey leaves) when TMV was inoculated to plants without antiserum treatment (Purdy, 1929). Although the data was not shown, ''preliminary experiments indicate that the antiserum to virus sap of tobacco is also capable of inactivating virus sap of tomato, pepper, and petunia plants'' supporting her idea that a discrete infectious agent was associated with tobacco mosaic disease. She had effectively used the tools of bacteriology, medical serology, and bioassay to ''test the power of inactivation of the antiserum'' to virus sap and ''the presence of an antibody, specific for virus itself'' (Purdy, 1929).

III. Beale's Later Work

Even with the changes at the Boyce Thompson Institute, Beale continued to do productive research, although the post-1932 work never came up to the importance of her earlier results. She published a series of papers using statistical analysis to quantitate TMV infections in tobacco (Beale, 1934b; Beale and Lojkin, 1944; Youden and Beale, 1934; Youden *et al.*, 1935). Bacteriologists and biologists routinely calculated the number of cells, spores, or colonies with microscopy and dilution plating. Beale brought serology, biochemistry, and virology together to use mathematical analyses for the quantitative measure of virus concentrations, instead of an empirical, applied approach. She used serum precipitin assays (taking only a few hours) instead of bioassays that required up to a week and facilities to grow tobacco plants, an observation that Stanley put to good use as he purified TMV (Creager, 2002; Stanley, 1941a). She also developed her ideas for applying serological techniques ''as an additional aid in the classification of filterable viruses in plants'' (Beale, 1934b). As part of developing standardized, quantitative methods, Beale designed a light box with the intent of more accurately reading precipitin tests (Beale, 1934a). Using serological techniques, she hoped to determine both the concentration of virus in a given sample and how a small percentage difference in virus concentrations could be measured (Beale, 1934b). She found that infectivity could be separated from antigenicity and that precipitin tests with dilution curves could show the relationship between the concentration of TMV and the number of local lesions (Beale, 1934b).

During this period, Beale also made two other observations that later became part of the standard tools of virology. The first was to improve Holmes' local lesion assay by modifying the inoculation technique and using a ''Latin square'' for statistical analysis of TMV concentrations. Beale determined that there was a logarithmic relationship between the number of local lesions on a plant and the dilution of the virus. From this she developed an equation, using her experience in bacteriology, to calculate virus titer with data from local lesion assays. Beale showed that the half-leaf method was the best strategy to compare two virus preparations (Youden and Beale, 1934; Youden *et al.*, 1935). She combined these techniques with precipitin-local lesion assays and statistical analysis to obtain ''more accurate estimates of the number of particles in the inoculum'' (Youden and Beale, 1934; Youden *et al.*, 1935), that is, to determine virus infectivity. Her ongoing interest to quantitatively measure the effects of viruses on plants was appreciated by scientists who came from a background in chemistry, but seems to have generally escaped the attention of plant pathologists.

In 1934, Beale also suggested that ''the specific antigenic material in Tobacco virus 1 [TMV] extract may be the virus itself'' (Beale, 1934b). She also believed that it was ''remarkable that the quantitative relation between antigen and active virus'' was so close (Beale, 1934b). Using light microscopy, she expanded on this using an earlier staining assay (Purdy, 1928c) to show that the crystalline inclusions in TMV-infected cells had physicochemical features that mimicked those of Stanley's crystals (Beale, 1937a,b; Stanley, 1935a, 1937). Epidermal peels of infected leaves showed that the hexagonal crystalline plates could be dissolved within 5 to 10 minutes into needle-like structures after addition of a drop a dilute acid of ~pH 1.3 (Beale, 1937a). This was also in agreement with results published by other TMV workers (Bawden *et al.*, 1936; Takahashi and Rawlins, 1937).

At the 1937 meeting of the American Association for the Advancement of Science, at the joint session with the Section on Botanical Sciences, Stanley discussed his ''Chemical Studies on the Virus of Tobacco Mosaic'' and Beale presented a paper on ''The Relationship of Intracellular Inclusions to Crystalline Tobacco Mosaic Virus Material.'' Beale had concluded that ''the intracellular crystalline deposits associated with tobacco mosaic disease are the source of Stanley's crystalline proteins obtained from tobacco virus extract'' (Beale, 1937a). As an internal control, she noted that the acid treatment did not affect the cell nucleus or x-bodies, and the crystalline plates were only observed in the chlorotic regions of the infected leaves (Beale, 1936, 1937a,b). Beale and Stanley's papers ''awakened great interest,'' one participant later reported. Both these and other virus papers indicated that ''recent work in the plant virus field is rapidly opening hopeful avenues of approach to some of nature's most perplexing problems'' (Ward, 1937).

Stanley believed ''that there is a real opportunity for cytological studies,'' in understanding the nature of TMV. He wrote that Beale's ''evidence for the relationship between the hexagonal crystals and virus was very good,'' in her ''excellent paper'' in the 1937 *Contributions from the Boyce Thompson Institute* (Stanley, 1941b). Beale's simple techniques using an epidermal peel of a TMV-infected tobacco leaf demonstrated that *in planta* crystals had the same physicochemical features as ''several hundred grams of crystalline virus'' prepared by Stanley after processing ''over five tons of diseased plants'' (Stanley, 1935b).

Although Beale had indirectly confirmed Stanley's results, K. Starr Chester, another Rockefeller scientist, had called into question the purity of Stanley's virus preparations. Chester used the Schultz–Dale reaction, a physiological test for anaphylaxis. For this assay, virgin female guinea pigs were sensitized with an antigen, in this case Stanley's TMV

crystals or healthy protein. If the antigen used for sensitization is present, the uterine horns of the guinea pig, composed of smooth muscle tissue, will contract, an event that is recorded on a kymograph drum (Chester, 1936). Chester wanted to show that healthy plant proteins were contaminants of Stanley's TMV crystals (Chester, 1936). This called into question Stanley's results, in particular, the isolation of TMV.

Chester's *Phytopathology* paper appears to have caused quite a stir, as evidenced by a letter that Elmer D. Merrill, the Administrator of Botanical Collections at Arnold Arboretum at Harvard University, sent to Kunkel in 1936. Merrill wrote that Stanley's work on isolating crystals that ''may prove to be the causative agent in mosaic diseases of plants is under fire'' (Merrill, 1936). In a reply to Merrill, Kunkel marshaled the work of Beale and her coworkers for a defense. Kunkel explained that two methods were accepted for determining the purity of a virus preparation ''the first is to inoculate plants, using the methods of Holmes and Price, and the second is to precipitate virus or some substance very closely associated with virus by means of immune rabbit serum, using the methods first described by Beale. As Dr Stanley's work progressed, it became necessary to make many measurements on the titer of samples. He made determinations by the plant method, but since several days were required to test the activity of any sample by this method, it seemed desirable for him to make use of the serological technique also. It required only a few minutes to make an activity measurement by the precipitin method'' (Kunkel, 1936).

In 1940 and 1941, Beale and Beatrice Seegal, who like Beale was an associate researcher in bacteriology at Columbia University, responded to Chester's claims with their own experimental results (Beale and Seegal, 1940, 1941). Beale and Seegal sensitized guinea pigs with normal tobacco protein and TMV, and did homologous and heterologous antigen challenges. They showed that the amounts of healthy protein needed to induce anaphylaxis were ''considerably greater than those reported by Chester'' and would ''certainly discourage the use'' of this anaphylaxis in the identification of ''traces of contaminating normal-plant-protein in virus preparations'' (Beale and Seegal, 1941).

Beale continued with her evaluation of the purity of virus preparations and the use of sera to characterize virus strains. For this she made serum to Holmes' ribgrass strain (producing ringspots on the host leaves), Holmes' masked strain (symptomless on tobacco) (Holmes, 1934), and several common strains of TMV, most of which were provided by Stanley as purified virus. But progress was slow at the Boyce Thompson Institute, especially in contrast to that of Stanley and

his group at the Rockefeller Institute who possessed the equipment, funding, and personnel to study viruses (Creager, 2002). When Stanley published electron micrographs showing the specificity of TMV antiserum to the purified virus (Stanley and Anderson, 1941) a new standard was set in defining the nature of TMV. From then on, local lesion assays and precipitin reactions would be only one of many tools in the virology armamentarium. The transition to new technology and techniques is reflected in Beale's correspondence with Stanley. In April 1941, she visited Stanley's lab to look at his ultracentrifuges. In a thank you note to Stanley she found the visit worthwhile, especially Max A. Lauffer's efforts in ''explaining their operation and usefulness'' (Beale, 1941). Beale added, ''I believe you agreed with me that, if possible, it would greatly facilitate the serologic project to have some sort of centrifuge, plants, and animals, under one roof if possible. I have not, however, forgotten your kind offer of cooperation in the matter of obtaining some of the virus-protein concentrates for immunization'' (Beale, 1941).

A *New York Times* article on women scientists in botany reported ''Dr Beale places in the same category the viruses responsible for plant and human ills and her research is directed with a view to advancing medical science by using the knowledge she has gained through plant experimentation'' (Anon, 1937). Yet Beale's progress toward this goal was disappointing, as made clear from a letter Stanley prepared in 1941 for her grant-in-aid application to the John and Mary R. Markle Foundation. He regarded her as an expert in serology, although the ''rapid advances that have been made in the chemical study of viruses during the past few years have unfortunately not been accompanied by similar advances in serological studies'' (Stanley, 1941c). Nevertheless, Beale received the grant, which was funded from 1942–1946. In 1942 she also was awarded a $125 grant-in-aid from the American Association for the Advancement of Science ''for a study of the photoelectric titration of plant viruses'' (Moulton, 1942).

Beale continued her work on quantitative serological assays to refine her analyses of virus concentrations and strains in concert with Holmes' local lesion assay (Beale and Lojkin, 1944, 1947). From these efforts she derived a formula, having transformed her data and those of others, to readily determine the amount of TMV in a given sample. She also estimated the concentration of TMV in solution using colorimetric assays. It was possible to differentiate between Holmes' ribgrass strain and the type strain of TMV, but the differences between the type strain and four other strains were not significant (Beale and Lojkin, 1947). Beale also showed that antibiotics did not control plant virus

infections. For instance, *Potato yellow dwarf virus*, a nucleorhabdovirus (Brakke *et al.*, 1950) on the systemic host *N. rustica*, and TMV on systemic and local lesion *Nicotiana* hosts were not inhibited by *in vivo* or *in vitro* applications of antibiotics including penicillin, chloramphenicol, and streptomycin (Beale and Jones, 1951). In a review on antibiosis and plant diseases, she considered that ''since the plant viruses are intracellular and multiply in a manner similar to obligate parasites, a logical approach to an effective method of control would seem to be some form of internal therapy'' (Weindling *et al.*, 1950). And she was hopeful that new antibiotics would be identified and ''prove to be valuable in the chemotherapeutic control of plant viruses'' (Weindling *et al.*, 1950).

In 1948, Beale was promoted to the rank of plant pathologist at the Boyce Thompson Institute. The same year, she ended her appointment as a research associate at Columbia University. Four years later she and her husband James Henry Beale (1885–1969), chief horticulturist at Boyce Thompson, retired to Ridgefield, Connecticut.

After her retirement, Beale began her monograph of the literature of plant viruses. She later recalled that the idea for this project extended back to 1932 when Kunkel gave Beale ''his card file on plant viruses'' when he left Boyce Thompson (Beale, 1976). Her research for the bibliography was funded by the American Tobacco Corporation, the Boyce Thompson Institute, and R. J. Reynolds Tobacco Company, grants from the National Institutes of Health and the National Library of Medicine, and support from the USDA and the Agricultural Research Service (Beale, 1976). In 1976, she completed the *Bibliography of Plant Viruses and Index to Research*, which was published by the Columbia University Press (Beale, 1976).

IV. Beale's Legacy

The *Bibliography of Plant Viruses and Index to Research* contained 29,000 plant virus/virology references from 1892–1970, and it reveals that the development of plant virology was a slow process in some cases. For example, in 1929 Beale had reported that TMV antiserum was specific and detected TMV *in planta*, showed the use of the complement fixation assay, precipitin test, absorption-precipitin test, and virus neutralization assay, and the usefulness of cross absorption with healthy plant sap. She had also described in detail the methodology associated with preparing plants, virus, antiserum, and setting up the antibody:antigen assays. What is discouraging is that the methodology did not become commonplace for several decades.

Her index held 1,257 citations on serology, less than 5% of all plant virus citations. Looking more carefully at the data, 31% of all serology citations were for TMV, *Potato virus X*, or *Potato virus Y*. From this it is clear that serology did not become a general technique to study plant viruses. Similarly, the use of purification techniques and electron microscopy had not really caught on, with 276 and 573 entries, respectively. As late as 1970, it seems that plant pathologists were still using the tools and methods of the early 20th century, including determining symptoms and host range to identify and characterize plant viruses.

That delay is particularly remarkable given that the essential tools for plant virology were published between 1927–1929, driven by three scientists: Helen Purdy Beale, H. H. McKinney, and F. O. Holmes. In 1927, McKinney, an iconoclastic USDA scientist, had elaborated his findings, which included the concepts of virus strains, cross protection, and that TMV strains were genetic mutants that developed in response to various pressures including host plants and environmental conditions (McKinney, 1929; Scholthof *et al.*, 1999). McKinney had developed the first ultracentrifugation techniques and reported that a high-speed pellet (50,000 rpm $\times$ 15 minutes) of TMV-infected plant sap was infectious (McKinney, 1927, 1972). Serology, local lesions, and centrifugation were the essential tools to determine the nature of TMV. Such techniques also had direct practical applications for crop improvement, epidemiology, and the push to the molecular biology of host-virus interactions (Creager, 2002; Creager *et al.*, 1999; Scholthof, 2004; Scholthof *et al.*, 1999). Today, serology remains a primary and powerful tool to diagnose plant viruses and to study the biology of host-pathogen interactions.

Kunkel's perspective on Beale's research bolsters the case that her findings were important. In 1933, he wrote: ''Mrs. Beale, who was with us at the Boyce Thompson Institute, has done considerable work on the precipitin reactions obtained in serum from rabbits sensitized to juice from tobacco and other plants having the tobacco mosaic disease. She is enthusiastic regarding possible applications of the method for measuring virus concentrations in unknown samples, early determination of the disease, diagnosis, etc. She has done her [Ph.D.] work under Dr Gay at Columbia University. He also seems enthusiastic as to the possibilities of this method of studying tobacco mosaic virus'' (Kunkel, 1933). And in 1952, in a letter of support for Beale's application for a Guggenheim Fellowship for the *Bibliography*, Kunkel wrote ''I have known Dr Helen Purdy Beale for about twenty-eight years. We were closely associated for several years in work on virus diseases of

plants at the Boyce Thompson Institute for Plant Research. In my opinion Dr Beale possesses unusual ability as a research worker and as a scholar. She was the first to show that certain plant viruses are antigenic. Her work in this field anticipated the later finding that these viruses behave in other respects like nucleoproteins. Dr Beale's researches on the use of serological and local-lesion methods in quantitative studies on tobacco-mosaic virus and other viruses was of a high order. She is one of those gifted persons who periodically comes forward with a new idea'' (Kunkel, 1952).

Yet her work was not absorbed into the literature of plant virology, as compared to Holmes' local lesion assay or McKinney's observations on cross protection. In 1970 Frederick C. Bawden in the *Annual Review of Phytopathology* wrote that in ''hindsight it is very evident'' that the techniques ''were even more valuable than those of us who used them appreciated'' (Bawden, 1970). He also was ''puzzled'' as to why so ''many virus workers long remained reluctant to use these invaluable techniques'' including general serology methods (Bawden, 1970). Two possible reasons for the belated reception of her work include Beale's lack of notoriety in the plant pathology and virology literature and the inherent technical difficulties associated with serology. Beale published the results of her experiments in the in-house *Contributions from the Boyce Thompson Institute* or as abstracts for scientific meetings instead of as peer-reviewed manuscripts. The failure to publish in the standard scientific journals in plant pathology and virology would have limited her exposure. In addition, significant expense and expertise were needed to adopt serology as a tool for standard practice. This was exemplified by the fact that almost 30 years after Beale's initial reports, Richard Matthews and Ellen M. Ball published the first step-by-step guides to serological techniques for plant virology (Ball, 1961; Matthews, 1957).

Beale died on November 5, 1976. Her obituary in the *Ridgefield Press* described her as ''unflappable, witty, and persevering'' (Anon, 1976). As a scientist, Beale contributed to the pioneering days of plant virology in the United States. Her insight and skillful laboratory technique brought immunology and serology to the plant virus community in the early 20th century, yet it was not until the 1960s that her tools came into general use for diagnostics and experimentation.

ACKNOWLEDGMENTS

We appreciate the critical and helpful comments provided by Herman Scholthof, Clay Griffith, Karl Maramorosch, and Angela Creager. K-BGS thanks Myron Brakke for

discussing the employment of women in plant pathology in the mid-20th century and to Al Scharen for the generous gift of H. H. McKinney's unpublished memoir. Thanks are extended to David Farrell for helping K-BGS in locating correspondence between Beale and Stanley at the Bancroft Library at UC-Berkeley and permission to quote materials. Donald Glassman of the Barnard College Archives was both gracious and helpful in locating materials related to Beale. We appreciate permission from the Rockefeller Archive Center for permission to quote materials from the L. O. Kunkel collection. Financial assistance was provided by the Aaron Morris and Anna E. Goldberg Memorial Fund (K-BGS). This manuscript was based in part, on a paper presented at the 2005 American Phytopathological Society annual meeting in Austin, Texas as part of a symposium entitled ''History of Virology: A Century of Developing a Discipline.''

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