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Unusual Two-Component Signal Transduction Pathways in the Actinobacteria

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I. Introduction

The actinobacteria are a group of high-GC Gram-positive bacteria which are of huge industrial importance and which also include many important human, plant, and animal pathogens. The best-studied genera in the actinobacteria are the corynebacteria, mycobacteria, and streptomycetes. The corynebacteria include the human pathogen *Corynebacterium diptheriae*, the causative agent of diptheria, and the important industrial organism *Corynebacterium glutamicum* which is used to produce around 1.2×10^6 and 6×10^5 tonnes of L-glutamate and L-lysine every year. The mycobacteria include the human pathogens *Mycobacterium tuberculosis*, which kills two million people in the world every year, and *Mycobacterium leprae*, the causative agent of Hansen's disease (leprosy). The streptomycetes are responsible for producing the majority of commercially important antibiotics as well as many important immunosuppressants, anticancer and antihelminthic drugs.

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The corynebacteria and mycobacteria are closely related, both belonging to the suborder *Corynebacterineae* within the order Actinomycetales (Stackerbrandt *et al.*, 1997). The streptomycetes are more distantly related but have been well studied because of their importance as producers of bioactive secondary metabolites and because of their interesting developmental life cycle, which includes both hyphal growth and sporulation (Chater, 2001).

In order to survive, all bacteria must sense and respond to their environment and one of the major ways in which they do this is via two-component signal transduction pathways. In the classical two-component system model, the extracellular domain of the transmembrane sensor kinase senses a specific signal, autophosphorylates its intracellular kinase domain, and passes that phosphate group to its cognate response regulator. The activated response regulator then switches on target genes to bring about a response to the original signal (Fig. 1). In bacteria, the range of environmental stimuli to which an organism can respond is said to be directly correlated to the number of sensor kinases encoded by that organism's genome (Kim and Forst, 2001).

The model organism for the actinobacteria, *Streptomyces coelicolor*, contains 164 two-component signal transduction genes, more than nearly any other bacterium. This includes 84 sensor kinase and 80 response regulator genes, 67 of which are adjacent on the chromosome and are predicted to form two-component systems (Hutchings *et al.*, 2004). The large number of two-component genes suggest that *S. coelicolor* is well adapted to its complex soil environment. In comparison, *M. tuberculosis* contains only 14 sensor kinases and 16 response regulators, 11 of which are paired on the chromosome (Tyagi and Sharma, 2004), while *C. glutamicum* contains 13 sensor kinases and 13 response regulator genes, all of which lie adjacent on the chromosome and presumably encode two-component systems (Kocan *et al.*, 2006). All of the genes encoding two-component systems in *M. tuberculosis* and *C. glutamicum* have been subjected to deletion mutagenesis. Only one system in *C. glutamicum, cgtSR4*, is essential for growth and viability (Kocan *et al.*, 2006). Similarly in *M. tuberculosis*, only a single gene, *mtrA*, which encodes a response regulator, is essential for viability. It could only be disrupted when a second copy was introduced *in trans* (Zahrt and Deretic, 2000). None of the other 29 two-component genes are essential in *M. tuberculosis*, although several have been implicated in intracellular survival and pathogenesis (Rison *et al.*, 2005).

Despite this comprehensive deletion analysis, very little is known about either the signals or the targets for the two-component signal transduction systems in either organism. In *M. tuberculosis*, signals

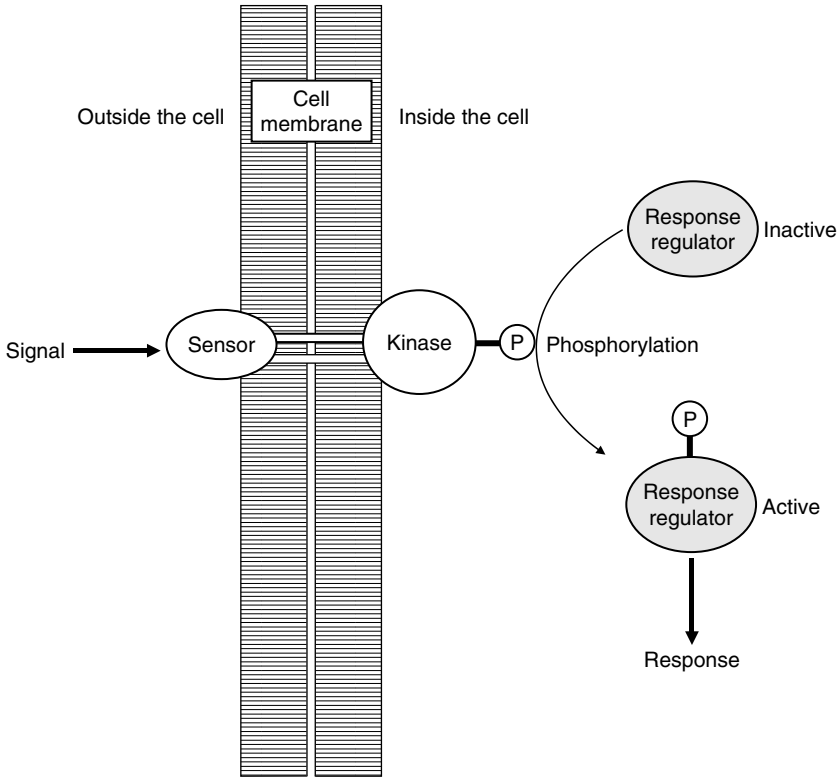

FIG. 1. The classical model of a bacterial two-component signal transduction pathway. The extracytoplasmic sensor domain of the transmembrane sensor kinase senses a specific signal, autophosphorylates its cytoplasmic kinase domain, and then passes that phosphate group to its cognate response regulator. The activated response regulator can then switch on target genes to bring about a response to the original signal.

have only been identified for one system, DosRS (Kendall *et al.*, 2004), while target genes have been identified for both DosRS and MtrAB (Table I). Like many of the two-component systems which have been characterized in the actinobacteria, neither system conforms to the classical model shown in Fig. 1. Other systems have been implicated in regulating genes required for virulence in *M. tuberculosis*, including MprAB (He *et al.*, 2006) and PhoPR (Walters *et al.*, 2006). In *C. glutamicum*, only the PhoRS and MtrAB two-component systems have been characterized. PhoRS senses limited phosphate availability and switches on phosphate starvation genes (Kocan *et al.*, 2006),

TABLE I

TWO-COMPONENT PROTEINS WHICH HAVE BEEN CHARACTERIZED IN THE MYCOBACTERIA, CORYNEBACTERIA, AND STREPTOMYCETES

Bacterium	Response regulator	Sensor kinase	Signal	Function	References
Mycobacterium tuberculosis	MtrA	MtrB	Unknown	Cell proliferation	Fol *et al.*, 2006
Mycobacterium tuberculosis	DosR	DosS, DosT	Hypoxia, nitric oxide, ethanol, H_2O_2	Dormancy	Sherman *et al.*, 2001; Kendall *et al.*, 2004
Mycobacterium tuberculosis	MprA	MprB	Detergents, alkaline pH	Cell envelope stress response	He *et al.*, 2006
Mycobacterium tuberculosis	PhoP	PhoR	Unknown	Cell envelope homeostasis	Walters *et al.*, 2006
Corynebacterium glutamicum	PhoR	PhoS	Phosphate starvation	Phosphate transport	Kocan *et al.*, 2006
Corynebacterium glutamicum	MtrA	MtrB	Unknown	Cell envelope stress, osmoprotection, and cell division	Moker *et al.*, 2004
Corynebacterium glutamicum	CitA	CitB	Citrate	Citrate uptake and metabolism	Kocan *et al.*, 2006
Streptomyces coelicolor	CseB	CseC	Cell envelope stress	Cell envelope homeostasis	Paget *et al.*, 1999b
Streptomyces coelicolor	VanR	VanS	Vancomycin	Vancomycin resistance	Hutchings *et al.*, 2006a

Streptomyces coelicolor	PhoR	PhoP	Phosphate starvation	Phosphate transport	Sola-Landa *et al.*, 2003
Streptomyces reticuli	SenR	SenS	Redox stress	Haemin resistance	Ortiz de Orue Lucana *et al.*, 2005
Streptomyces coelicolor	AbsA2	AbsA1	Unknown	Antibiotic production	Adamidis *et al.*, 1990
Streptomyces coelicolor	AfsQ2	AfsQ1	Unknown	Antibiotic production	Ishizuka *et al.*, 1992
Streptomyces coelicolor	CutR	CutS	Unknown	Antibiotic production	Tseng and Chen, 1991
Streptomyces thermoviolaceus	ChiR	ChiS	Chitinobiose	Chitinase expression	Tsujibo *et al.*, 1999
Streptomyces coelicolor	KdpE	KdpD	Osmotic stress	Potassium transport	Hutchings *et al.*, 2004
Streptomyces coelicolor	OsaA	OsaB	Osmotic stress	Osmosensing	Bishop *et al.*, 2004
Streptomyces coelicolor	WhiI	Unknown	Unknown	Development	Ainsa *et al.*, 1999
Streptomyces coelicolor	RamR	Unknown	Unknown	Development	O'Connor *et al.*, 2002
Streptomyces coelicolor	BldM	Unknown	Unknown	Development	Molle and Buttner, 2000
Streptomyces coelicolor	RedZ	Unknown	Unknown	Antibiotic production	Guthrie *et al.*, 1998
Streptomyces coelicolor	GlnR	Unknown	Unknown	Glutamine synthesis	Wray and Fisher, 1991

while MtrAB is homologous to the *M. tuberculosis* system and is implicated in the regulation of cell division, osmoprotection, and cell envelope integrity (Moker *et al.*, 2004; Table I).

Carrying out comprehensive deletion analysis of the *S. coelicolor*, two-component systems is more challenging simply because of the large number of genes involved. Of the 164 predicted two-component system proteins, only 15 have been assigned functions or phenotypes (Table I). Many of these were identified prior to the completion of the genome sequence through classical genetic screens for mutants which affect either antibiotic production or development. Others, such as KdpDE and PhoRP, were identified in *S. coelicolor* on the basis of homology to systems which are widespread in bacteria. The PhoRP system senses phosphate starvation and switches on the expression of alkaline phosphatase (Sola-Landa *et al.*, 2003), while KdpDE senses turgor pressure (Jung and Altendorf, 2003) and activates expression of the KdpFABC potassium transporter which adjusts the potassium content of the cytoplasm to maintain turgor pressure (Wood, 1999).

II. Unusual Two-Component Systems in Actinobacteria

In recent years, it has become clear that not all two-component systems conform to the classical model shown in Fig. 1. Variations on the components themselves include bifunctional sensor kinases, which can act as both kinase and phosphatase. It has been shown that disruption of a bifunctional sensor kinase gene can lead to cross-talk between the response regulator and noncognate sensor kinase(s) or to phosphorylation by a small molecule phosphodonor such as acetyl phosphate. Branched pathways, in which a single sensor kinase can activate multiple response regulators, or where multiple sensor kinases can feed into a single response regulator have also been reported, as have ''three-component systems'' in which the activity of either the sensor kinase or its cognate response regulator is modulated by an accessory protein. Many of these systems have been identified in the actinobacteria and these are the main focus of this chapter.

A. VanRS and Vancomycin Resistance

S. coelicolor and *Streptomyces toyocaensis* contain a cluster of genes, *vanRSJKHAX*, which confer high-level inducible resistance to glycopeptide antibiotics (Hong *et al.*, 2004; Pootoolal *et al.*, 2002). These genes are split into four transcription units, the *vanRS* operon, the *vanJ* and *vanK* genes, and the *vanHAX* operon (Hong *et al.*, 2004). *vanRS* encodes the

VanRS two-component system which controls expression of the *vanRSJ-KHAX* gene cluster. In *S. coelicolor*, the VanS sensor kinase is activated by the glycopeptide antibiotic vancomycin, resulting in autophosphorylation of the cytoplasmic kinase domain and subsequent phosphorylation of VanR. Phospho-VanR then switches on the expression of the *vanRSJKHAX* genes (Hong *et al.*, 2004, Hutchings *et al.*, 2006a).

Vancomycin works by binding to the D-Ala-D-Ala termini of the stem peptides of cell wall precursors to prevent cross-linking of the mature cell wall. The *vanHAX* genes encode enzymes which reprogram the cell wall such that the precursors terminate D-Ala-D-Lac, a substrate for which vancomycin has 1000-fold lower specificity (Bugg *et al.*, 1991). *vanJ* and *vanK* are novel genes that are only found in the vancomycin resistance gene clusters of streptomycetes. The function of *vanJ* is unknown but it is not required for vancomycin resistance (Hong *et al.*, 2004). Disruption of *vanK*, however, resulted in a vancomycin-sensitive strain. Subsequent work revealed that *vanK* encodes a Fem protein which catalyses the addition of a single glycine crossbridge amino acid to the D-Ala-D-Lac-containing cell wall precursors. This crossbridge is required for cross-linking of the mature cell wall. Under normal growth conditions, an essential enzyme called FemX adds the glycine crossbridge to D-Ala-D-Ala-containing precursors, but FemX cannot recognize precursors containing D-Ala-D-Lac. Instead, in the presence of vancomycin, VanK is expressed and replaces the essential FemX enzyme. This led to the discovery that the essential *femX* gene could be disrupted in the presence of vancomycin, or any structurally related glycopeptide which induces VanS activity (Hong *et al.*, 2005). Furthermore, suppressor mutations, which allowed the *femX* strain to grow in the absence of glycopeptides, were all mapped to the *vanS* gene, leading to the discovery that a null mutation in *vanS* results in constitutive vancomycin resistance (Hutchings *et al.*, 2006a). This is because, in the absence of inducer, VanR is phosphorylated by the small molecule phosphodonor, acetyl phosphate and VanS acts as a VanR-specific phosphatase to suppress the levels of phospho-VanR (Hutchings *et al.*, 2006a). When cells are exposed to vancomycin, VanS switches to a kinase and phosphorylates VanR, resulting in rapid induction of the *van* genes (Fig. 2).

B. AbsA1/2

The AbsA1/2 two-component system was identified through the analysis of mutations that blocked production of the pigmented antibiotics actinorhodin and undecylprodigiosin in *S. coelicolor*

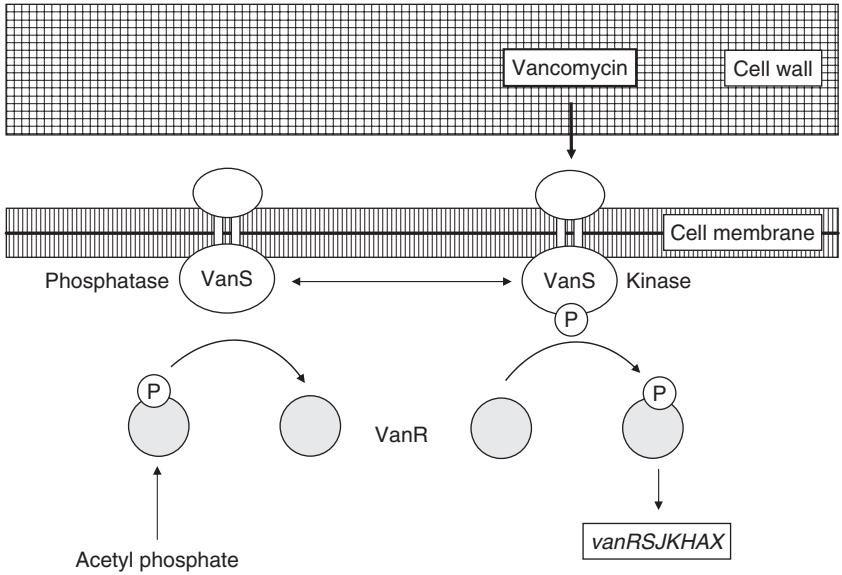

FIG. 2. The bifunctional VanRS two-component system. In the absence of the activating signal, the sensor kinase, VanS, acts as a specific phosphatase to suppress the levels of phosphorylated response regulator, VanR, and keep target genes switched off. Nonspecific phosphorylation of VanR arises via the small molecule phosphodonor acetyl phosphate. In the presence of the activating signal, the activity of the sensor kinase switches from phosphatase to kinase and VanS then actively phosphorylates VanR, which in turn activates expression of the vancomycin resistance genes. When the signal disappears, the activity of the VanS reverts to a phosphatase and rapidly switches off the response.

(Adamidis *et al.*, 1990). The point mutations accounting for this phenotype were all located in the *absA1* (sensor kinase) gene, whereas deletion of either *absA1* or *absA2* resulted in hyperproduction of actinorhodin and undecylprodigiosin, known as the *p*recocious *h*yperproduction of *a*ntibiotics (Pha) phenotype (Brian *et al.*, 1996). This implies that the original point mutants were gain-of-function mutations that led to constitutive phosphorylation of the response regulator and that phosphorylated AbsA2 turns off antibiotic production. Mutation of the conserved H202 residue, the proposed site of autophosphorylation, resulted in a strain exhibiting the Pha phenotype, although the strain produced even more undecylprodigiosin and actinorhodin than the *absA1* deletion strain (Sheeler *et al.*, 2005). This could be explained by the fourfold increase in AbsA2-specific phosphatase activity of the AbsA1 H202A protein,

compared to the wild-type protein, *in vitro* (Sheeler *et al.*, 2005). If AbsA2 is phosphorylated *in vivo* by either acetyl phosphate or a noncognate sensor kinase then the overproduction of pigmented antibiotics in the H202A *absA1* strain could be explained by its enhanced phosphatase activity (Sheeler *et al.*, 2005).

C. SenRS and Haemin Resistance

The SenRS two-component system negatively regulates the expression of *furS, cpeB*, and *hbpS* in *Streptomyces reticuli*. The *furS* gene encodes a zinc-dependent transcription factor, *cpeB* encodes a catalase-peroxidase, and *hbpS* encodes a novel haem-binding protein (Ortiz de Orue Lucana *et al.*, 2005). HbpS is an extracellular protein that is secreted via the twin arginine translocase (Tat) pathway (Ortiz de Orue Lucana *et al.*, 2004), a pathway typically used to transport fully folded proteins and those requiring cofactor assembly in the cytoplasm (Sargent *et al.*, 2006). Furthermore, it has been demonstrated that the sensor kinase, SenS, binds to HbpS *in vitro* suggesting HbpS is an accessory protein to the sensor kinase (Ortiz de Orue Lucana *et al.*, 2005). Disruption of *hbpS* reduces the expression of the *furS-cpeB* operon implying that HbpS negatively modulates the activity of SenS. The SenS protein has five predicted transmembrane helices at the N-terminus and no obvious sensor domain (Ortiz de Orue Lucana *et al.*, 2005). HbpS might therefore interact with one or more of the short extracellular loops of the protein to modulate its activity. Both *senRS* and *hbpS* mutants are more sensitive to hydrogen peroxide and haemin and it has been proposed that this system senses redox stress (Ortiz de Orue Lucana *et al.*, 2005). If this is the case then it seems likely that the haem-containing HbpS acts as a separate sensor module for the SenRS two-component system. Reduction of the haem group of HbpS could block SenS and thereby inactivate the SenRS signal transduction system, resulting in derepression of the *furS-cpeB* operon. The resulting expression of the CpeB catalase-peroxidase would confer resistance to hydrogen peroxide and other redox stresses.

D. The σ^E-CseABC Signal Transduction Pathway

The *sigE* operon encodes σ^E, an extracytoplasmic function (ECF) RNA polymerase sigma factor, CseA, a lipoprotein, CseB, a response regulator, and CseC, a sensor kinase (Paget *et al.*, 1999a). Null mutations in *sigE* and *cseB* have identical phenotypes, namely an altered

cell wall profile and hypersensitivity to lysozyme (Paget *et al.*, 1999b), suggesting that the *sigE* promoter is the only target for CseB. To our knowledge, this is the only case in which a two-component system is solely dedicated to regulating the expression of an RNA polymerase sigma factor. The signal sensed by CseC has yet to be elucidated but there is good evidence that this sensor kinase responds to cell envelope damage since expression of *sigE* can be induced by a wide range of cell wall-specific compounds, including antibiotics such as vancomycin and bacitracin and muramidases such as lysozyme (Hong *et al.*, 2002). Interestingly, while null mutations have been made in the *sigE, cseA*, and *cseB* genes, all attempts to disrupt *cseC* have been unsuccessful, suggesting that CseC is essential (M. I. Hutchings, unpublished data). This implies that CseB can be activated via cross-talk in the absence of CseC and that the resulting overexpression of σ^{E} is lethal to the cell. The targets for RNA polymerase holoenzyme containing σ^{E} are largely unknown but it seems likely that these targets will include genes involved in cell wall homeostasis.

CseA, a novel lipoprotein with no homologues outside the streptomycetes, acts as a negative regulator of *sigE* expression (Hutchings *et al.*, 2006b; Fig. 3). Lipoproteins are anchored in the membrane by a lipid anchor such that all of the protein is located outside of the cell and it has been proposed that, in Gram-positive bacteria, these proteins are equivalent to periplasmic proteins in Gram-negative bacteria (Nielsen and Lampen, 1982). The mechanism by which CseA negatively modulates *sigE* expression is not yet clear but the fact that CseA is extracellular strongly suggests that it blocks signal sensing by CseC (Hutchings *et al.*, 2006b). If CseA downregulates transcription of *sigE* by modulating the activity of the sensor kinase, CseC, this is not unprecedented. Several two-component systems of Gram-positive bacteria are known to be regulated by extracytoplasmic accessory proteins, including the KapB lipoprotein of *Bacillus subtilis*, which is proposed to act as an accessory protein to the KinB sensor kinase involved in triggering sporulation (Dartois *et al.*, 1997). Other examples include SenS, which is regulated by the Tat-secreted protein HbpS (Section II.B) and YycH, a membrane-anchored protein which regulates the essential envelope stress-sensing system YycFG from the extracytoplasmic face of the membrane (Szurmant *et al.*, 2005).

Bioinformatics analysis of the genome sequence of *S. coelicolor* revealed five other sensor kinase genes which form operons with genes encoding putative lipoproteins. Four of these are also linked to response regulator genes, presumably forming two-component systems (Fig. 4). However, while the five sensor kinase proteins are all ~40% identical to

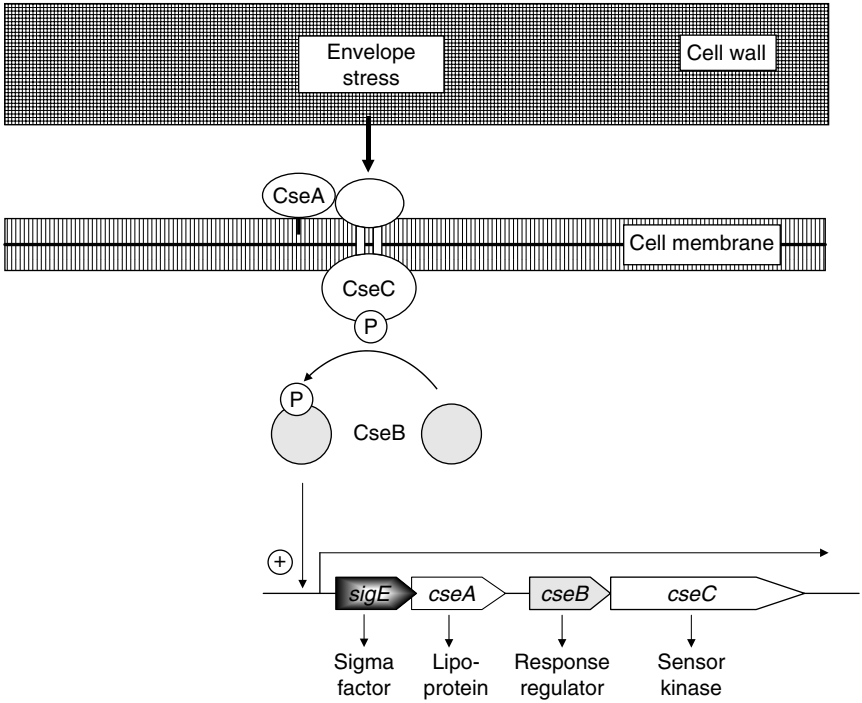

FIG. 3. Model for the σ^E cell envelope stress response in *Streptomyces coelicolor*. The *sigE-cseABC* operon of *S. coelicolor* encodes an RNA polymerase sigma factor σ^E, a lipoprotein CseA, a response regulator CseB, and a sensor kinase CseC (Cse, *c*ontrol of *s*igma *E*). CseC senses cell envelope stress and phosphorylates CseB which then activates expression of the *sigE* operon. RNA polymerase holoenzyme containing σ^E is proposed to activate genes involved in maintaining cell envelope homoeostasis. The novel lipoprotein, CseA, negatively modulates transcription of *sigE* from the extracytoplasmic face of the membrane, probably by blocking signal sensing by CseC.

CseC, the five lipoproteins do not share significant identity with CseA. The *sigE* operon is also the only operon in *S. coelicolor* to encode both a sigma factor and a two-component system. One of the other five operons, the *afsQ* operon, is divergently transcribed from a sigma factor gene, *sigQ*, whose product is closely related to σ^E (Fig. 4). However, expression of *sigQ* is not regulated by the AfsQ system, at least under the conditions tested (M. I. Hutchings, unpublished data). The function of these systems is unknown, but one operon, SCO3011-SCO3013, is an orthologue of the *mtrAB-lpqB* operon of *M. tuberculosis*.

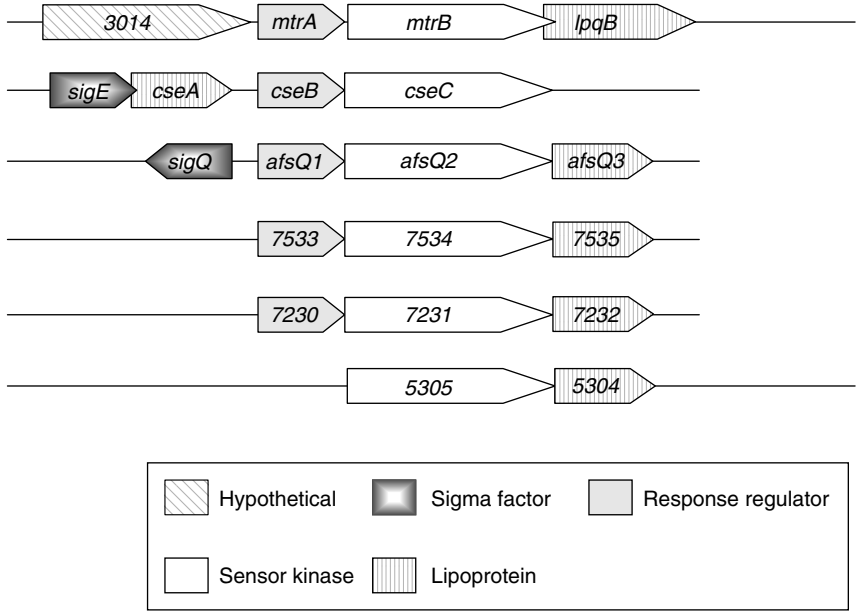

FIG. 4. Two-component system operons that also encode lipoproteins in *Streptomyces coelicolor*. In *S. coelicolor*, a subgroup of six closely related sensor kinases are encoded by operons which also encode lipoproteins. The activity of at least one, CseC, and possibly all of these sensor kinases is modulated by its cotranscribed lipoprotein. Genes without names are identified by their SCO numbers (http://www.sanger.ac.uk/Projects/S_coelicolor/). There is no response regulator gene associated with the SCO5304-5 operon.

E. MtrAB

MtrA is an essential response regulator in the human pathogen *M. tuberculosis.* It is encoded within the three-gene operon *mtrAB-lpqB*, which also encodes its cognate sensor kinase, MtrB, and a lipoprotein of unknown function, LpqB. This operon is conserved in all of the actinobacterial genomes published to date, with the exception of the obligate intracellular human pathogen *Tropheryma whipplei* (Hoskisson and Hutchings, 2006), a bacterium which has undergone extensive gene decay (Bentley *et al.*, 2003). It is significant that the *mtrAB-lpqB* genes are conserved as functional genes in *M. leprae* since this bacterium also has a greatly reduced genome and is predicted to have lost most of the sigma factor and two-component genes found in the other mycobacteria (Tyagi and Saini, 2004). Furthermore, MtrA protein was detected by two-dimensional gel electrophoresis of soluble cell extracts

from *M. leprae* (Marques *et al.*, 1998), showing that MtrA is not only conserved but is also expressed in *M. leprae.*

In *M. tuberculosis*, it has been shown that MtrA binds to, and activates, expression of the *dnaA* gene (Fol *et al.*, 2006). This is the first direct MtrA target to be identified in the actinobacteria and reveals a possible role for MtrA in cell cycle progression. The *dnaA* gene, which encodes the master regulator of DNA replication, is an essential gene in all bacteria. If it is absolutely dependent on MtrA for activation in *M. tuberculosis* this would explain why disruption of MtrA is lethal. Interestingly, MtrB is nonessential in *M. tuberculosis* suggesting that MtrA can be phosphorylated via cross-talk in the absence of its cognate sensor kinase (Fol *et al.*, 2006).

In the closely related *Mycobacterium avium* disruption of the *mtrB* gene resulted in a strain which is fivefold more sensitive to penicillin, ciprofloxacin, and clanthromycin. The *mtrB* mutant also exhibited cell envelope defects, resulting in increased permeability and an elongated cell shape (Cangelosi *et al.*, 2006). All of these phenotypes are in agreement with those reported for *mtrAB* mutants of *C. glutamicum* (Moker *et al.*, 2004).

It is significant that *mtrA* is essential in mycobacteria but not in the closely related corynebacteria. Microarray studies comparing wild-type *C. glutamicum* with an *mtrAB* strain revealed that many osmoprotection genes were downregulated, while a protease/protease inhibitor pair was upregulated in the *mtrAB* null mutant. Overexpression of this protease, MepA, in *E. coli* (but not *C. glutamicum*) led to an elongation of the cells and led the authors to suggest that this protein has a role in regulating cell wall turnover (Moker *et al.*, 2004).

In *S. coelicolor*, a fourth gene, *SCO3014*, lies just five base pairs upstream of *mtrA* and preliminary analysis of the transcriptional organization of these genes suggests that *SCO3014, mtrA, mtrB*, and *lpqB* are transcriptionally coupled in *S. coelicolor* (Tri-Than Ngat and M. I. Hutchings, unpublished data). *SCO3014* encodes a homologue of a eukaryotic translation initiation factor and is only linked to the *mtrAB-lpqB* genes in *Streptomyces.* The function of this gene is unclear but null mutations in *SCO3014, mtrB*, and *lpqB* all affect normal septation and sporulation. Attempts to disrupt *mtrA* have so far been unsuccessful, suggesting this may also be an essential gene in *Streptomyces* (M. I. Hutchings, unpublished data).

Disruption of MtrAB affects cell cycle progression in all of the actinobacteria studied to date. This could explain both the essential nature of MtrA in the mycobacteria and the reason for MtrAB conservation throughout this group of high-GC Gram-positive bacteria. The most

likely hypothesis appears to be that MtrAB senses and responds to general stresses which could ultimately inhibit cell division. It is also likely that the conserved LpqB lipoprotein, which was described as a signature protein of the actinobacteria (Gao *et al.*, 2006) plays a role in signal transduction through MtrB.

F. Intramembrane-Sensing Histidine Kinases

Mascher *et al.* (2003) used whole genome microarrays to study vancomycin- and bacitracin-induced cell envelope stress in the model low-GC Gram-positive bacterium *B. subtilis.* They identified three two-component systems, YvqEC, YvqQ/YvcP, and BceRS which could sense and respond to such stress. They noted that all three sensor kinases have extracellular domains less than 20 residues long and proposed that such proteins constitute a new family of intramembrane-sensing histidine kinases which sense signals generated within the cell membrane (Mascher *et al.*, 2003). These kinases appear to be widespread in the low-GC Gram-positive bacteria known as the *Firmicutes* and they are often genetically linked to ABC transporters which are known, or proposed, to be involved in antibiotic resistance (Mascher *et al.*, 2003).

In the actinobacteria, intramembrane-sensing histidine kinases are found only in the genomes of *S. coelicolor*, *Streptomyces avermitilis*, and *Bifidobacterium longum* (Table II). The functions of these proteins are unknown but one, SCO1369, is genetically linked to a three-gene operon encoding an putative ABC transporter. This suggests that it too might be involved in antibiotic resistance. Another, SCO3390, is encoded within an operon which also encodes a *tmrB*-like gene. TmrB confers resistance to the antibiotic tunicamycin, an inhibitor of the essential cell wall assembly enzyme MraY (Brandish *et al.*, 1996). The surrounding genes of the other kinases do not give any clues to their function and it will be interesting to investigate whether they really are involved in sensing cell envelope stress.

G. Leaderless Transcripts

Leaderless transcripts are those which start at the same point as the translational start codon, usually the adenine nucleotide of the ATG start codon. Such leaderless transcription appears to be a common theme in *S. coelicolor* two-component system operons, and is more common in actinobacteria as a whole than in other bacteria (Day and Jansenn, 2004). The reason why some genes have leaderless transcripts

TABLE II

INTRAMEMBRANE-SENSING HISTIDINE KINASES IN THE ACTINOBACTERIA

Organism	Gene number	Length (amino acids)[a]		
		Whole protein	Sensor domain	N-terminus
Streptomyces coelicolor	SCO1369	404	11	91
Streptomyces coelicolor	SCO3390	401	5	61
Streptomyces coelicolor	SCO3740	370	7	89
Streptomyces coelicolor	SCO5282	375	7	81
Streptomyces coelicolor	SCO5784	358	3	87
Streptomyces coelicolor	SCO6163	303	3	44
Streptomyces coelicolor	SCO6424	331	3	64
Streptomyces avermitilis	SAV2971	368	6	76
Streptomyces avermitilis	SAV7391	388	8	84
Bifidobacterium longum	BL1001	358	1	75

[a]Mascher *et al.* (2003) stipulated that the total length of the protein must not exceed 400-amino acid residues, with an N-terminus not greater than 100 residues and an extracellular domain (the part of the protein between the two transmembrane helices) not longer than 20 residues. We have relaxed the criteria slightly to include SCO1369 and SCO3390 as their adjacent genes suggest they could be involved in the envelope stress response.

is not yet clear but it has been demonstrated *in vitro* that dissociation of the 70S ribosome is not required in order for leaderless transcripts to be translated (Udagawa *et al.*, 2004). This suggests that these genes need to be expressed very rapidly in response to an activating signal and therefore ''jump the queue'' at the ribosome. The mechanism by which these transcripts bind to the ribosome, however, is not yet known.

In *M. tuberculosis*, the *mtrA* transcript is leaderless (Via *et al.*, 1996) and in *S. coelicolor*, four out of the five two-component system operons (*vanRS, afsQ1/2, phoPR*, and *absA1/2*) that have been mapped have leaderless transcripts (Anderson *et al.*, 2001; Hong *et al.*, 2004; Ishizuka *et al.*, 1992; Sola-Landa *et al.*, 2003). The exception is *cseBC* in which both genes appear to be under the control of the *sigE* promoter. This leaderless arrangement, along with the positive autoregulation of most two-component system operons, probably allows rapid expression and amplification of the signal transduction cascade in response to the specific environmental signals inducing each system.

III. Orphan Two-Component Proteins

C. glutamicum is unusual among the actinobacteria in containing only paired two-component system genes (Kocan *et al.*, 2006). *M. tuberculosis* contains two unpaired sensor kinase genes and six orphan response regulator genes of which only one, the unpaired sensor kinase DosT, has been assigned a function (Roberts *et al.*, 2004). *S. coelicolor* contains 17 unpaired sensor kinase genes and 13 orphan response regulator genes. Five of these orphan response regulators have been implicated in the regulation of developmental processes in *S. coelicolor* or in the regulation of primary or secondary metabolism (Table I). It is interesting to note that in the differentiating bacterium *Caulobacter crescentus*, developmental processes are also regulated by orphan response regulators, although these were shown to form cognate pairs with sensor kinases encoded elsewhere on the chromosome (Skerker *et al.*, 2005).

Four of the 13 orphan regulators in *S. coelicolor* are missing the conserved aspartate residue which is the site of phosphorylation, suggesting they have an alternative mechanism of activation, or are phosphorylated on a different active site residue (Hutchings *et al.*, 2004) while another, BldM, has a normal phosphorylation pocket but is active *in vivo* in the absence of phosphorylation (Molle and Buttner, 2000). The high sensor kinase to response regulator ratio in *S. coelicolor* suggests that some response regulators are activated by multiple sensor kinases. Such branched arrangements allow a single regulon to be switched on by multiple signals, as has been demonstrated in other branched systems. For example, DosRST in *M. tuberculosis* (Section III.A). Alternatively, in *M. tuberculosis*, the response regulators exceed sensor kinases in number suggesting either that a single sensor kinase feeds into more than one response regulator, that the orphan response regulators are phosphorylated by a small molecule phosphodonor such as acetyl phosphate, or that they are not dependent on phosphorylation for activity.

A. DosRST: A Branched Pathway

DosRS (also known as DevRS) is the only two-component system in *M. tuberculosis* for which inducing signals have been identified. It is also the only branched pathway to be characterized in the actinobacteria. The *dosRS* genes are upregulated in response to hypoxia, nitric oxide (NO), ethanol, and hydrogen peroxide (Kendall *et al.*, 2004; Sherman *et al.,* 2001). The sensor kinase, DosS, is predicted to be cytoplasmic and is divided into a C-terminal kinase domain and an

N-terminal sensor domain. The N-terminus contains two GAF domains which are predicted to be involved in signal sensing. The proximal GAF domain has been shown to bind haem *in vitro*, and is presumed to sense oxygen and NO directly but the function of the second GAF domain is not yet known (Sardiwal *et al.*, 2005).

Disruption of the *dosR* gene resulted in a strain with a severe defect in hypoxic viability, while disruption of *dosS* had very little effect on hypoxic survival (Sherman *et al.*, 2001). This led to the discovery that a second sensor kinase, Rv2027c, which is 62.5% identical to DosS can also activate DosR (Roberts *et al.*, 2004; Saini *et al.*, 2004). This kinase, renamed DosT, also contains two GAF domains but is expressed constitutively under hypoxic conditions. It has been proposed that DosS and DosT sense the same signals, with DosT sensing them first and activating DosR which then switches on expression of *dosRS* to amplify the hypoxic response. A *dosST* double mutant has the same phenotype as a *dosR* mutant, suggesting that these are the only activators of DosR (Roberts *et al.*, 2004).

The majority of genes in the DosR regulon encode hypothetical or conserved hypothetical proteins (Sherman *et al.*, 2001). However, the regulon includes six proteins which contain the Universal stress protein domain (Usp) and another protein, α-crystallin or Acr, which belongs to the small heat shock protein family (Sherman *et al.*, 2001; Tyagi and Sharma, 2004). These proteins typically act as chaperones and Acr is proposed to have a role in latent infection by *M. tuberculosis* by slowing the growth of *M. tuberculosis* immediately following infection (Hu *et al.*, 2006).

B. Predictions Based on Homology and Ontology

In *S. coelicolor*, some predictions can be made about which sensor kinases feed into which response regulators based on genome location and sequence similarity (Table III). For example, one response regulator, SCO4596, falls into an operon with two sensor kinases, SCO4597 and SCO4598, which share 52% identity over the total length of the protein. It is likely that both kinases feed into this response regulator. Interestingly, alignment of these sensor kinases reveals that the main difference in sequence is in the N-termini of the proteins, which are predicted to contain the sensor domain. The N-termini share 45% identity and the C-termini, containing the kinase domains, share 79% identity. This suggests a model whereby each kinase is activated by a different environmental signal, autophosphorylates and passes that phosphate to a single response regulator so that the net response

TABLE III

PREDICTED BRANCHED PATHWAYS IN *S. COELICOLOR*

Response regulator	Putative sensor kinases (% identity)		
SCO0203	SCO0204 (100)	SCO0211 (49)	SCO3948 (39)
SCO4596	SCO4597 (100)	SCO4598 (52)	
OsaB	OsaA (100)	SCO7327 (60)	

is the same. SCO7327 and OsaA are both yeast-like osmosensing sensor kinases which share 60% identity over the total length of the proteins. OsaA activates OsaB in response to osmotic stress (Bishop *et al.*, 2004) and it seems likely that SCO7327 also feeds into OsaB. Finally, the response regulator SCO0204 shares 49% identity with *M. tuberculosis* DosR, while SCO0203 and SCO3948 share 35% identity with DosS and DosT, respectively. Like DosS, both sensor kinases contain two GAF domains at their N-termini (Sardiwal *et al.*, 2005). Another sensor kinase, SCO0211, shares 36% identity with the kinase domain of DosS but lacks the N-terminal, GAF-containing, sensor domain. All three sensor kinases are predicted to be soluble, cytoplasmic proteins and may be involved in the survival response of *S. coelicolor* to microaerobic and anaerobic conditions (van Keulen *et al.*, 2003).

C. TYPICAL RESPONSE REGULATORS

The N-termini of response regulators typically contain several conserved residues which, together, form the phosphorylation pocket. These residues include two adjacent aspartates at the N-terminus of the protein, an aspartate in the middle of the N-terminal domain which is the site of phosphorylation, a hydroxylated residue at position 82, normally serine or threonine, and a lysine residue at the end of the N-terminus, close to position 105. Typical response regulators contain all of the conserved phosphorylation pocket residues. Atypical response regulators are those which lack two or more of the conserved residues which make up the phosphorylation pocket (Hutchings *et al.*, 2004). In *M. tuberculosis*, all six orphan response regulators contain atypical phosphorylation pockets, suggesting they might be activated by an alternative mechanism. To date, nothing is known about the function of these proteins. Of the 13 orphan response regulator genes in the *S. coelicolor* genome, seven have typical phosphorylation pockets, five have atypical phosphorylation pockets, and a single protein,

BldM, has a pseudo-phosphorylation pocket. BldM has a typical phosphorylation pocket but is active *in vivo* in the absence of phosphorylation (Molle and Buttner, 2000). Only two, RamR and GlnR, out of seven typical response regulators in *S. coelicolor*, have been studied experimentally and these are discussed briefly below.

1. *RamR*

RamR regulates expression of the *r*apid *a*erial *m*ycelium (*ramCSAB*) operon in *S. coelicolor*. The *ramC* gene encodes the SapB synthetase RamC, *ramS* encodes the SapB lantibiotic-like peptide precursor, and the *ramAB* genes encode the subunits of an ABC transporter. SapB functions as a biological surfactant, breaking the surface tension at the air–water interface thus allowing the aerial hyphae to grow up into the air. RamC, which was previously thought to be a serine–threonine kinase, acts as a lantibiotic synthase and catalyses the formation of the SapB lantibiotic from the peptide precursor (Kodani *et al.*, 2005).

2. *GlnR*

The *S. coelicolor* genome contains five glutamine synthetase (GS) homologues: a GSI of the β-subtype, three of the GSI-α-subtype, and a GSII-type enzyme, a type normally associated with eukaryotes (Brown *et al.*, 1994). The GlnR response regulator is required for transcription of the GSI-like enzyme, *glnA*, in *S. coelicolor* (Fink *et al.*, 2002; Wray and Fisher, 1991) and a *glnR* null mutant is a glutamine auxotroph. A close homologue, named GlnRII, is required for GSII transcription. GlnRII shows significant similarity to GlnR in the C-terminal DNA-binding domain but lacks the N-terminal CheY domain for sensor kinase interaction, suggesting that GlnRII is not a true response regulator.

D. Atypical Response Regulators

1. *BldM*

The original point mutations isolated in the *bldM* locus lay in the putative phosphorylation pocket and resulted in a strain which could not form aerial hyphae (Molle and Buttner, 2000). However, replacement of the conserved aspartate-54 with unphosphorylatable residues such as asparagine or alanine did not affect its function, with wild-type levels of aerial hyphae and spores still being formed (Molle and Buttner, 2000). In *E. coli*, substitution of the conserved aspartate to asparagine in the response regulator CheY resulted in an active protein *in vivo* as a result of phosphorylation of an adjacent serine residue

(Appleby and Bourret, 1999; Bourret *et al.*, 1990). Phosphorylation at alternative residues has also been reported in FixJ of *Rhizobium meliloti* and NtrC of *E. coli* (Moore *et al.*, 1993; Reyrat *et al.*, 1994). However, no potentially phosphorylatable hydroxyamino acid residues are found adjacent to D54 in BldM, suggesting that BldM has a pseudo-phosphorylation pocket.

2. *WhiI*

whiI encodes an orphan response regulator that is required for normal septum formation early in the development of aerial hyphae. A *whiI* null mutant is unable to sporulate and the lack of gray spore pigment gives it a *whi*te colony phenotype. The phosphorylation pocket of WhiI lacks the lysine residue and an aspartate which are conserved in conventional phosphorylation pockets. This suggests that *whiI* is regulated by alternative mechanism, such as small ligand binding, or interaction with another protein (Ainsa *et al.*, 1999).

3. *RedZ*

RedZ, the pathway-specific activator for undecylprodigiosin (also known as Red) production, lacks the paired aspartate residues and the lysine residue conserved in the phosphorylation pocket of conventional response regulators (Guthrie *et al.*, 1998) and the mechanism of activation is not known. However, there is some experimental evidence which suggests that RedZ could be regulated posttranslationally (J. White and M. J. Bibb, personal communication).

IV. Conclusions and Perspectives

The unusual mechanisms of action described here are likely to be widespread in the bacterial kingdom. Bioinformatics analysis of all the complete bacterial genome sequences suggest that many two-component systems are encoded by gene clusters which also encode extracellular proteins (G. Chandra and M. I. Hutchings, unpublished data). In Gram-positive bacteria, these are usually anchored in the membrane, either by lipid modification or by a single, N-terminal, transmembrane helix. Several examples of two-component accessory proteins have been described (Section II.D). In Gram-negative bacteria such accessory proteins are usually localized to the periplasm, for example CpxP of *E. coli*, which regulates the envelope stress-sensing sensor kinase, CpxA (Isaac *et al.*, 2005). It appears that these accessory proteins can either negatively regulate the sensor kinase, by preventing unnecessary activation of the signal transduction pathway in the absence of a *bona fide* signal, or

positively regulate the sensor kinase by directing the signal to its sensor domain.

In recent years, it has also become clear that many sensor kinases are bifunctional. This gives the sensor protein much tighter control over the whole signal transduction pathway because, instead of simply phosphorylating its cognate response regulator and letting it go, the sensor kinase can also suppress the levels of phosphorylated response regulator in the absence of signal and switch the pathway off when the signal has disappeared. It seems likely that, as in the case of VanS and MtrB, removing these bifunctional kinases from the cell will result in constitutively active response regulators. The consequences of this could be catastrophic, because all of the intracellular targets of that regulator would be constitutively expressed.

Although progress in characterizing signals and targets for two-component systems has been slow, with the advent of genome sequencing, whole-genome microarrays, and proteomics technology, the speed of this progress should increase dramatically. To date, most of the detailed characterization of these systems has been carried out in *E. coli* and *B. subtilis*. However, interest in the actinobacteria is increasing rapidly as the need for new antibiotics, and for novel treatments of persistent mycobacterial infections, becomes ever more desperate. Two-component systems are implicated both in the production of secondary metabolites in streptomycetes and in the persistent infections of mycobacteria. It has also been noted that essential two-component proteins in pathogenic bacteria, such as MtrA in *M. tuberculosis*, are potential targets for new classes of antibiotic since these systems are widespread in bacteria but are absent from the animal kingdom as a whole (Wolanin *et al.*, 2002).

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Acyl-HSL Signal Decay: Intrinsic to Bacterial Cell–Cell Communications

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I. Introduction

Many members of the bacterial phylum *Proteobacteria* employ low-molecular-weight chemical signaling molecules in the coordination of their group behaviors. This process of cell–cell communication is known as quorum sensing and it was first described as the mechanism that underlies light production by the squid symbiont *Vibrio fischeri* (Nealson and Hastings, 1979; Nealson *et al.*, 1970). The structure of the signaling molecule of *Vibrio fischeri*, *N*-3-(oxohexanoyl) homoserine lactone (3OC6HSL), was determined (Eberhard *et al.*, 1981) and was the first representative of a large family of molecules, the acyl-homoserine lactones (acyl-HSLs). These dedicated signaling molecules all have an HSL moiety but can differ in the substituents and length of the acyl chain, which can vary from 4 to 16 carbons (Fuqua *et al.*, 2001). Their function as signaling molecules employed by diverse Gram-negative bacteria has been studied extensively. The canonical way in which acyl-HSLs are utilized in quorum sensing requires the synthesis of acyl-HSL signals by synthases, which are encoded by homologues of the *luxI* gene and signal response regulator proteins, which are

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encoded by homologues of the *luxR* gene (Engebrecht *et al.*, 1983; Gambello and Iglewski, 1991; Piper *et al.*, 1993). The LuxR protein homologues bind to acyl-HSL-signaling molecules and activate the transcription of genes that have proven advantages to express when cell numbers are high. The way these molecules function to coordinate group behaviors at the genetic and biochemical levels has been intensively studied over the last 30 years. The synthesis of acyl-HSLs (Hanzelka *et al.*, 1997; Schaefer *et al.*, 1996), interactions with response regulators (Stevens and Greenberg, 1997), acyl-HSL quorum-sensing controlled regulons (Wagner *et al.*, 2003), and, in recent years, the degradation of acyl-HSL-signaling molecules have been investigated. The latter topic is the subject of this chapter.

Acyl-HSL quorum signal degradation is important given that the presence and concentration of these signaling molecules are key to several microbial group behaviors. Acyl-HSL-mediated quorum sensing has been found to underlie a host of microbial group behaviors from antibiotics and toxins production to swarming motility and biofilm formation (Fuqua *et al.*, 2001; Swift *et al.*, 2001). Quorum sensing would not be effective as a gene regulation mechanism if signaling molecule concentrations did not accurately portray cell numbers. The critical concentration for activation of quorum responses varies for different quorum-sensing microbes from ca. 5 nM to 2 μM *in vitro* (Fuqua *et al.*, 1995; Kaplan and Greenberg, 1985; Pearson *et al.*, 1995; von Bodman *et al.*, 1998; Whiteley *et al.*, 1999). Since the quorum-sensing process depends on the concentration of signaling molecules accurately reflecting cell population density, signal molecule stability and potential for degradation are key areas of study if we aim to understand how this process functions in nature.

II. Acyl-HSL-Degrading Organisms, Enzymes, and Homologues

Since the year 2000 with the first reports of microbially mediated signal degradation (Dong *et al.*, 2000; Leadbetter and Greenberg, 2000), the search and study of microbes and organisms that engage in acyl-HSL signal degradation has been fruitful (Table I). Database searches have identified numerous homologues of known acyl-HSL lactonase and acylase enzymes in a wide range of species suggesting that this activity could be widespread (Table II). Organisms with homologues to known acyl-HSL-degrading enzymes hail from all three domains of life (*Bacteria, Eucarya*, and *Archaea*) and dwell in a wide range of environments and conditions, from mesophilic and mesothermic to extreme haloalkaliphilic, thermophilic, and acidophilic environments, suggesting that within a wide range of environments there may be biotic

TABLE I

Demonstrated Mechanisms and Proteins Involved in Acyl-HSL Degradation by Diverse Bacteria and Eukaryotes

	Species	Mechanism	Protein	References
Bacteria				
Proteobacteria				
α-Proteobacteria	*Agrobacterium tumefaciens* A6	Lactonase	AttM	Zhang *et al.*, 2002
	Agrobacterium tumefaciens C58	Lactonase	AttM and AiiB	Carlier *et al.*, 2003
β-Proteobacteria	*Variovorax paradoxus* VAI-C	Acylase	ND*	Leadbetter and Greenberg, 2000
	Ralstonia sp. XJ12B	Acylase	AiiD	Lin *et al.*, 2003
γ-Proteobacteria	*Pseudomonas aeruginosa* PAO1	Acylase	PvdQ/PA2385 and QuiP/PA1032	Huang *et al.*, 2003, 2006
	Pseudomonas sp. PAI-A	Acylase	ND	Huang *et al.*, 2003
	Klebsiella pneumoniae KCTC2241	Lactonase	AhlK	Park *et al.*, 2003
	Acinetobacter sp. C1010	ND	ND	Kang *et al.*, 2004
Firmicutes Bacilli	*Bacillus* sp. 240B1	Lactonase	AiiA	Dong *et al.*, 2001, 2000
(Low-G + C Gram-positive)	*Bacillus thuringiensis*	Lactonase	AiiA homologues	Dong *et al.*, 2002; Lee *et al.*, 2002
	Bacillus cereus	Lactonase	AiiA homologues	Dong *et al.*, 2002; Reimmann *et al.*, 2002
	Bacillus mycoides	Lactonase	AiiA homologues	Dong *et al.*, 2002
	Bacillus stearothermophilus	Lactonase	ND	Park *et al.*, 2003
	Bacillus anthracis	Lactonase	AiiA homologues	Ulrich, 2004

(*continued*)

TABLE I (*Continued*)

	Species	Mechanism	Protein	References
Actinobacteria Actinobacteria (High-G + C Gram-positive)	*Rhodococcus erythropolis* W2	Acylase and oxidoreductase	ND	Uroz *et al.*, 2003, 2005
	Arthrobacter sp. IBN110	Lactonase	AhlD	Park *et al.*, 2003
	Streptomyces sp. M664	Acylase	AhlM	Park *et al.*, 2005
Eukarya	Mammalian sera/tissues	Lactonase	Paraoxonases (PON1, PON2, and PON3)	Chun *et al.*, 2004; Draganov *et al.*, 2005; Ozer *et al.*, 2005; Yang *et al.*, 2005
	Alga *Laminaria digitata*	Oxidized halogen reaction	Not identified	Brochardt *et al.*, 2001; Michels *et al.*, 2000
	Legume *Lotus corniculatus*	ND	ND	Delalande *et al.*, 2005

*ND, not determined.

TABLE II

HOMOLOGUES OF ACYL-HSL LACTONASES AND ACYLASES

Organism with homologue	Protein (as annotated)	AA similarity (%)	Acyl-HSLase homologue	Phylum
Acyl-HSL lactonase homologues				
Deinococcus radiodurans R1	Hypothetical protein	57	AiiB	*Deinococcus-Thermus*
Moorella thermoacetica ATCC 39073	β-lactamase-like	53	AiiB	*Firmicutes*
Thiomicrospira crunogena XCL-2	β-lactamase-like	51	AiiB	*Proteobacteria*
Bradyrhizobium japonicum USDA 110	AttM/AiiB family protein	51	AiiB	*Proteobacteria*
Natronomonas pharaonis	Hydrolase	50	AiiB	*Euryarchaeota*
Photorhabdus luminescens subsp.	Hypothetical protein plu2238	88	AttM	*Proteobacteria*
Yersinia intermedia ATCC 29909	Zn-dependent hydrolases	87	AttM	*Proteobacteria*
Nocardioides sp. JS614	β-lactamase-like	56	AttM	*Actinobacteria*
Clostridium beijerincki NCIMB 8052	Putative metallohydrolase	56	AhlD	*Firmicutes*
Staphylococcus haemolyticus	Hypothetical protein	52	AhlD	*Firmicutes*
Aspergillus oryzae	Hypothetical protein	49	AhlD	*Fungi*
Haloarcula marismortui	Hypothetical protein	45	AhlD	*Euryarchaeota*
Sulfolobus solfataricus	Conserved hypothetical protein	55	AhlD	*Crenarchaeota*
Thermoplasma volcanium	Zn-dependent hydrolase	50	AhlD	*Euryarchaeota*
Paracoccus denitrificans PD1222	β-lactamase-like	56	AiiA	*Proteobacteria*
Archaeoglobus fulgidus DSM 4304	Hypothetical protein	54	AiiA	*Euryarchaeota*
Desulfovibrio desulfuricans	Metallo-β-lactamase family	52	AiiA	*Proteobacteria*
Bacillus licheniformis ATTC 14580	Putative hydrolase	51	AiiA	*Firmicutes*

(*continued*)

TABLE II (*Continued*)

Organism with homologue	Protein (as annotated)	AA similarity (%)	Acyl-HSLase homologue	Phylum
Leptospira interrogans sv. Copenhageni	Metallo-β-lactamase	51	AiiA	*Spirochaetes*
Ralstonia eutropha JMP134	β-lactamase-like	51	AiiA	*Proteobacteria*
Frankia sp. EAN1pec	β-lactamase-like	50	AiiA	*Actinobacteria*
Rubrobacter xylanophilus DSM 9941	β-lactamase-like	50	AiiA	*Actinobacteria*
Deinococcus geothermalis DSM 11300	β-lactamase-like	50	AiiA	*Deinococcus-Thermus*
Rhizobium etli	Hypothetical protein	49	AiiA	*Proteobacteria*
Canis familiaris (dog)	Serum paraoxonase/arylesterase	91	PON1	*Metazoa*
Bos taurus (cow)	Similar to paraoxonase 1	90	PON1	*Metazoa*
Oryctolagus cuniculus (rabbit)	Serum paraoxonase/arylesterase 1	90	PON1	*Metazoa*
Tetraodon nigroviridis (pufferfish)	Unnamed protein product	78	PON1	*Metazoa*
Xenopus laevis (African clawed frog)	MGC80011 protein	77	PON1	*Metazoa*
Streptomyces coelicolor	Gluconolactonase precursor	53	PON1	*Actinobacteria*
Trichodesmium erythraeum	Senescence marker protein-30	53	PON1	*Cyanobacterium*
Aspergillus fumigatus (green mold)	Hypothetical protein	50	PON1	*Fungi*
Homo sapiens (human)	Paraoxonase/arylesterase	94	PON2	*Metazoa*
Pan troglodytes (chimpanzee)	Similar to paraoxonase/arylesterase	94	PON2	*Metazoa*
Gallus gallus (domestic chicken)	Similar to paraoxonase 2	84	PON2	*Metazoa*

Pongo pygmaeus (Bornean orangutan)	Paraoxonase 1	83	PON2	*Metazoa*
Xenopus tropicalis (western clawed frog)	Paraoxonase 1	80	PON2	*Metazoa*
Platichthys flesus (wild flounder)	Paraoxonase (arylesterase)	73	PON2	*Metazoa*
Danio rerio (zebra fish)	Similar to paraoxonase 2	72	PON2	*Metazoa*
Strongylocentrotus purpuratus (urchin)	Similar to paraoxonase 2	54	PON2	*Metazoa*
Caenorhabditis briggsae (nematode)	Hypothetical protein CBG10483	50	PON2	*Metazoa*
Acyl-HSL acylase homologues				
Deinococcus radiodurans R1	Aculeacin A acylase	68	AiiD	*Deinococcus-Thermus*
Hahella chejuensis KCTC 2396	Protein related to penicillin acylase	51	AiiD	*Proteobacteria*
Nocardioides sp. JS614	Peptidase S45, penicillin amidase	52	AiiD	*Actinobacteria*
Psychrobacter cryohalolentis K5	Peptidase S45, penicillin amidase	53	AiiD	*Proteobacteria*
Ralstonia metallidurans	Penicillin amidase	81	AiiD	*Proteobacteria*
Ralstonia solanacearum GMI1000	Conserved hypothetical protein	89	AiiD	*Proteobacteria*
Rubrivivax gelatinosus PM1	Protein related to penicillin acylase	48	QuiP	*Proteobacteria*
Azotobacter vinelandii AvOP	Peptidase S45, penicillin amidase	82	QuiP	*Proteobacteria*
Gloeobacter violaceus PCC 7421	Probable penicillin amidase	49	QuiP	*Cyanobacteria*
Nostoc punctiforme PCC 73102	Related to penicillin acylase	48	QuiP	*Cyanobacteria*
Pseudomonas fluorescens PfO-1	Penicillin amidase family protein	79	QuiP	*Proteobacteria*
Pseudomonas putida	Penicillin amidase family protein	69	PvdQ	*Proteobacteria*
Pseudomonas syringae	Penicillin amidase family protein	74	PvdQ	*Proteobacteria*
Streptomyces lavendulae subsp.	Penicillin V acylase precursor	52	PvdQ	*Actinobacteria*
Actinoplanes utahensi	Aculeacin A acylase	54	AhlM	*Actinobacteria*
Shewanella baltica OS155	Peptidase S45, penicillin amidase	48	AhlM	*Proteobacteria*

interactions with acyl-HSLs. A variety of these interactions involve acyl-HSLs in host–symbiont relationships (Nealson *et al.*, 1970) and pathogenic infections (de Kievit and Iglewski, 2000; Parsek and Greenberg, 2000). Given such diverse and close interactions, it is perhaps not surprising that eukaryotic hosts have evolved mechanisms to interact with acyl-HSLs (Chun *et al.*, 2004; Telford *et al.*, 1998). Acyl-HSL-degrading activity by paraoxonase (PON) enzymes was discovered from mammalian cells in epithelial and colon cells, which are cells in the front lines of contact with potential pathogens (Chun *et al.*, 2004). Many species containing such homologues reside in environments for which known acyl-HSL producers have been found. The colocalization of acyl-HSL-producing and acyl-HSL-degrading organisms in environments suggests the range of community interactions that could exist. Acyl-HSL production by a haloalkaliphilic archaeon that activates an *Agrobacterium* biosensor is believed to regulate the production of an extracellular protease (Paggi *et al.*, 2003). Other haloalkaliphilic species are known to have acyl-HSL-degrading lactonase homologues: *Natronomonas* sp. and *Haloarcula* sp. (Table II). Also the discovery of nine long-chain acyl-HSLs produced by the acidophilic archaeon *Acidithiobacillus ferrooxidans* (Farah *et al.*, 2005) provides evidence for the presence of acyl-HSLs in acidic environments. Given the greater stability of long-chain acyl-HSLs under acidic conditions (Yates *et al.*, 2002), these molecules would be stable in this environment and could be subject to degradation by other organisms such as *Thermoplasma* or *Sulfolobus* sp., which can exist in such environments and have known acyl-HSL-degrading enzyme homologues. These locations would be interesting environments to study microbial production and degradation of acyl-HSLs.

Several methods have been successful to identify organisms that degrade acyl-HSLs from the environment, including enrichment cultures which select organisms that utilize acyl-HSLs as the sole source of carbon and energy (Huang *et al.*, 2003; Leadbetter and Greenberg, 2000), as well as the screening techniques by which environmental isolates are either grown initially in undefined medium followed by examination of cultured organisms for acyl-HSL-degrading abilities via bioassay (D'Angelo-Picard *et al.*, 2005) or incubated initially in acyl-HSL signal followed by screening based on bioassay or growth on signal (Uroz *et al.*, 2003). These methods have identified bacteria that degrade signal via an acylase mechanism, by which the acyl-HSL is cleaved at the amide bond (Leadbetter and Greenberg, 2000; Uroz *et al.*, 2003), or that utilize the lactonase mechanism, by which the microbes hydrolyze the HSL ring (Park *et al.*, 2003) (Fig. 1). As more techniques

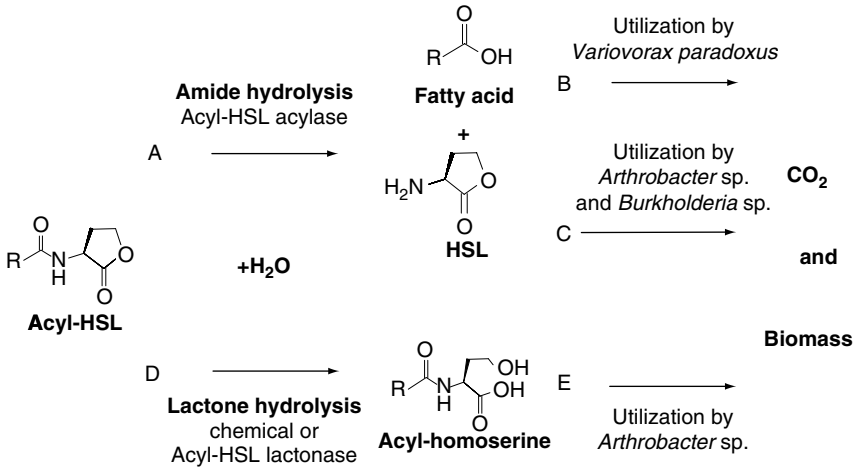

FIG. 1. Key mechanisms by which acyl-HSLs can be inactivated and further degraded. (A) Cleavage of the amide bond by bacterial AHL acylase yields the corresponding fatty acid and HSL (Leadbetter, 2001; Lin *et al.*, 2003). The acyl-HSL amide is chemically stable under conditions of nonextreme temperature and pH. (B) The fatty acid that is released is known to be utilized by organisms such as *Variovorax paradoxus* (Leadbetter and Greenberg, 2000). (C) The HSL that is released is known to be utilized as an energy nutrient by diverse *Arthrobacter* and *Burkholderia* sp. (Yang *et al.*, 2006). (D) Cleavage of the lactone ring by bacterial AHL lactonase yields the corresponding acyl-homoserine (Dong *et al.*, 2000; Park *et al.*, 2003; Zhang *et al.*, 2002). The lactone ring is also subject to chemical hydrolysis; the chemical half-life of the ring is ca. $10^{[7-\mathrm{pH}]}$ days (Eberhard *et al.*, 1981; Schaefer *et al.*, 2000). (E) The acyl-homoserine degradation product generated by lactonolysis is known to be utilized as an energy nutrient by *Arthrobacter* sp. (Flagan *et al.*, 2003).

are devised to screen acyl-HSL-degrading organisms in the soil rhizosphere (Jafra and van der Wolf, 2004; Uroz *et al.*, 2003) and other environments, the challenge will be to understand how acyl-HSL degradation is used by the microbes identified and to examine their degradation abilities in light of the biology of the natural communities in which these microbes reside.

The ability of both acyl-HSL-degrading acylase and lactonase enzymes to interfere with the quorum sensing of pathogens that employ acyl-HSL signals in pathogenicity or virulence has been tested in a number of ways: coculture of acyl-HSL-degrading and acyl-HSL-producing bacteria (Dong *et al.*, 2000; Park *et al.*, 2003; Uroz *et al.*, 2003), expression of acyl-HSL-degrading enzymes in quorum-sensing pathogens (Dong *et al.*, 2000; Lin *et al.*, 2003; Reimmann *et al.*, 2002),

and expression of acyl-HSL-degrading enzymes in transgenic plant hosts (Dong *et al.*, 2001). These methods have been effective to attenuate pathogenicity of acyl-HSL-utilizing pathogens such as *Erwinia* and *Pseudomonas* sp. When an extracellularly excreted acyl-HSL acylase enzyme of *Streptomyces*, AhlM, was added to *Pseudomonas aeruginosa* cultures, decreased virulence factors were observed (Park *et al.*, 2005). Expressing an acyl-HSL lactonase homologue of *Bacillus* sp., AiiA, in *Pseudomonas aeruginosa* prevented accumulation of C4HSL and reduced the accumulation of 3OC12HSL, and thus reduced virulence gene expression and swarming motility of *Pseudomonas aeruginosa* (Reimmann *et al.*, 2002). Expression of AiiA in a non-acyl-HSL-degrading soil bacterium, *Pseudomonas fluorescens*, reduced potato soft rot as a preventative against infection and as a curative measure; the recombinant strains also interfered with *Pseudomonas chlororaphis* which produces compounds effective as a natural biocontrol against fungal pathogens (Molina *et al.*, 2003). Applications of acyl-HSL-degrading enzymes have been reviewed extensively (Dong and Zhang, 2005).

Signal degradation via the lactonase mechanism by *Agrobacterium tumefaciens* (Zhang *et al.*, 2002) demonstrates how this process can be an integral part of an acyl-HSL-mediated quorum-sensing circuit. AttM, the acyl-HSL-degrading lactonase of *Agrobacterium tumefaciens*, is growth-phase regulated and controls the bacterium's exit from its quorum state, making this process an essential component of the microbe's quorum-sensing system (Zhang *et al.*, 2002). It is possible that bacteria such as *Ralstonia* sp. which contain homologues to the acyl-HSL acylase AiiD, or *Pseudomonas aeruginosa*, which quorum senses with two known acyl-HSL-mediated quorum-sensing systems and has two acyl-HSL-degrading acylases (Huang *et al.*, 2003, 2006), may also utilize acyl-HSL degradation in the modulation of their quorum-sensing systems. Inactivation by eukaryotes infected by bacterial pathogens that employ quorum sensing in their virulence is a new area of study for which regulation of this activity will be interesting and important.

III. Mechanisms of Acyl-HSL Degradation

Over the past 6 years, diverse acyl-HSL degradation mechanisms have been documented. Besides chemical hydrolysis, the rates of which are subject to pH, temperature and acyl-HSL side chain structure, many bacteria and even eukaryotes are found to be able to rapidly degrade acyl-HSL via different mechanisms (Table I).

A. Chemical Hydrolysis

In the environment, the rings of acyl-HSLs decompose chemically under alkaline conditions (Voelkert and Grant, 1970). The lactone ring is hydrolyzed, generating the corresponding acyl-homoserine.

1. *pH-Dependent Chemical Hydrolysis*

The Eberhard equation is prevailingly accepted to determine the half-lives of acyl-HSLs at different pHs: acyl-HSL $t_{1/2} = 10^{[7-\mathrm{pH}]}$ days (Eberhard *et al.*, 1981; Schaefer *et al.*, 2000). For instance, when studying the acyl-HSL concentration variation during growth of a plant pathogen *Erwinia carotovora* subsp. *carotovora*, 3OC6HSL decreased ca. 18.7% after 9 hours into stationary phase in LB medium amended with 100-mM potassium phosphate buffer to maintain pH at ca. 6.7, which was slightly higher than that calculated using Eberhard's equation (12.2%) (Byers *et al.*, 2002).

Since LB is poorly buffered, the pH of aerobically grown cultures of both *Pseudomonas aeruginosa* (which employs both C4HSL and 3OC12HSL as its quorum-sensing signals) and *Yersinia pseudotuberculosis* (which employs 3OC6HSL and C6HSL) increased from ca. 7.0 to greater than 8.5 after 24 hours of growth (Yates *et al.*, 2002). Under such conditions, acyl-HSLs become largely degraded due to pH-dependent hydrolysis. After synthetic 3OC12HSL was incubated with stationary-phase cell-free culture supernatant of *Pseudomonas aeruginosa*, high performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS) revealed the corresponding hydrolyzed product of 3-oxododecanoyl-homoserine. Furthermore, by acidifying the culture supernatants to pH 2.0, recycling of the lactone ring of C4HSL was observed. However, ^{13}C nuclear magnetic resonance (NMR) spectroscopy showed that such ring reformation was not a simple reversal of the ring cleavage process. The pH must first approach the pK of the carboxyl group (ca. pH 2) before the hydroxyl group can close the ring. Ring recycling has also been observed by Uroz *et al.* (2005).

A similar pattern of decreasing half-life with increasing pH was observed by Delalande *et al.* (2005); however, the half-lives were much shorter at low pHs yet much longer at high pHs than those predicted by the Eberhard equation. For instance, at 20°C, the half-life was only 8 days at pH 5.5 as opposed to 30 days theoretically, while it was 12 hours at pH 8.0 as opposed to 2 hours theoretically.

2. *Temperature-Dependent Hydrolysis*

Formerly reported, 3OC6HSL is temperature stable and may be boiled without loss of activity (Schaefer *et al.*, 2000) and can even endure heating at 140°C in air for 10 min (Eberhard *et al.*, 1981) as a thin dry film. In contrast, Byers *et al.* (2002) found that 3OC6HSL was subject to rapid inactivation by boiling. Substantial loss of the molecule (ca. >50%) was observed when synthetic 3OC6HSL was boiled for 10 min. The reason for such contradiction was probably because the latter experiment was performed in aqueous solutions where hydrolysis could readily occur.

In addition, nonenzymatic temperature-dependent hydrolysis was demonstrated at different temperatures (Yates *et al.*, 2002). The experiments were carried out at given temperature with known quantities of acyl-HSLs hydrolyzed in unbuffered aqueous solutions. The resulted pH decrease was measured, from which the concentration of released corresponding acyl-homoserine and thus the acyl-HSL-releasing rate were calculated. Much greater hydrolysis was observed at 37°C than at 22°C with all the acyl-HSLs tested (C4HSL, C6HSL, 3OC6HSL, and C8HSL) albeit to different extents. A similar pattern of such half-life decreasing as temperature increases was also observed by Delalande *et al.* (2005).

On the other hand, it is well accepted that acyl-HSLs should be stored at low temperature (i.e., –20°C) (Schaefer *et al.*, 2000). The 3OC6HSL concentration was found to be the same when assayed 1 day or 1 week after being stored at –20°C (Byers *et al.*, 2002). Acyl-HSLs degradation rates appear to be greatly reduced at such low temperature.

3. *Acyl-HSL Chain Structure-Dependent Hydrolysis*

When investigating temperature influence on acyl-HSL hydrolysis rates as described above, Yates *et al.* (2002) found out that the rates also depended on the side chain length and substitution at the third carbon position. For the four acyl-HSLs tested (C4HSL, C6HSL, 3OC6HSL, and C8HSL), at both 22°C and 37°C, the longer the acyl side chain, the slower the hydrolysis was, while 3OC6HSL hydrolyzed faster than C6HSL which had no substitution at the third carbon position. Consistently, by using ^{13}C NMR spectroscopy, the authors found that the ring opening of HSL, C3HSL, and C4HSL decreased accordingly. As pointed out by the authors, the lactone ring is made less electrophilic because the carbonyl group gains more electrons as the acyl chain length increases, and thus less susceptible to attack by hydroxide ions. While an oxo-substitution at the third carbon position does the opposite.

B. Biochemical Hydrolysis by Acyl-HSL Lactonases

That acyl-HSLs can also be degraded biochemically was first reported in 2000 by a protein designated AiiA, produced by *Bacillus* sp. (Dong *et al.*, 2000). The enzymatic activity was later shown to be that of an acyl-HSL lactonase (Dong *et al.*, 2001) using a mechanism similar to chemical hydrolysis, cleaving the ester bond of the lactone ring, and releasing the corresponding acyl-homoserine (Fig. 1D).

The purified AiiA protein from the *Firmicute* bacterial isolate *Bacillus* sp. 240B1 effectively inactivated three acyl-HSLs tested, that is, 3OC6HSL, 3OC8HSL, and 3OC10HSL (Dong *et al.*, 2000). By using HPLC and ESI-MS, digestion of 3OC6HSL with AiiA resulted in only one product of the open-ring form of the molecule, that is, 3-oxohexanoyl-homoserine (Dong *et al.*, 2001). Similar results were observed with all the acyl-HSLs tested (C4HSL, 3OC8HSL, and 3OC12HSL). Following this discovery, 20 bacterial isolates among 800 from soil and plant samples were found capable of enzymatic degradation of acyl-HSLs (Dong *et al.*, 2002). Eight isolates were identified belonging to *Bacillus thuringiensis*, with acyl-HSL-inactivating activities ranging from 480 to 680 pmol hour^{-1} per unit of OD_{600}. Nine genes, exhibiting high levels of homology to *aiiA*, were cloned from strains belonging to *Bacillus thuringiensis, Bacillus cereus*, and *Bacillus mycoides*. Two *Bacillus* sp. A23 and A24 have also been isolated from rhizosphere soils, whose 16S rRNA genes are >99% identical with those from *Bacillus cereus* group, and identified encoding acyl-HSL lactonase (*aiiA* homologues) (Reimmann *et al.*, 2002). They both degraded synthetic C4HSL and C6HSL rapidly and almost completely. Recombinant *Pseudomonas aeruginosa* PAO1 expressing the *aiiA* homologue from A24 dramatically reduced 3OC12HSL and completely prevented C4HSL accumulation, thus markedly decreasing expression of several virulence factors and swarming motility. In addition, *aiiA* homologue genes were found in 16 subspecies of *Bacillus thuringiensis*, of which all wild-type strains showed acyl-HSL-degrading activities albeit to different extent (Lee *et al.*, 2002). The *Bacillus anthracis* AiiA lactonase has also been shown capable of efficiently cleaving acyl-HSLs (Ulrich, 2004).

The acyl-HSL degradation enzyme encoded by *attM*, a homologue of *aiiA*, has been identified in *Agrobacterium tumefaciens* A6 (Zhang *et al.*, 2002). By using HPLC and ESI-MS, the only reaction product of 3OC8HSL digested by purified AttM was determined to be 3-oxooctanoyl-homoserine, thus confirming AttM to be an acyl-HSL lactonase. Furthermore, *attM* is negatively controlled by a transcription factor, AttJ.

Expression of *attM* is growth phase dependent, which is initially suppressed by AttJ but enhanced substantially at stationary phase with 3OC8HSL largely degraded. Therefore, *Agrobacterium tumefaciens* A6 adopts a unique signal turnover system to exit from conjugation-related quorum sensing. Besides *attM*, another *attM*-paralogous gene, *aiiB*, has been identified in *Agrobacterium tumefaciens* C58 (Carlier *et al.*, 2003). Serving as a lactonase as well, *aiiB* is encoded on pTi plasmid, as apposed to *attM* lying on pAt plasmid. It was observed that AiiB was less efficient than AttM in degrading synthetic acyl-HSLs (i.e., C6HSL, 3OC6HSL, C7HSL, C8HSL, and 3OC8HSL). Recombinant *Erwinia carotovora* subsp. *atroseptica* expressing either *attM* or *aiiB* reduced accumulation of its cognate quorum-sensing signal 3OC8HSL to two to three orders of magnitude less than wild type with AttM being more effective. Interestingly, *attM*, encoded by the *attKLM* operon, was further demonstrated to be involved in an assimilative pathway of γ-butyrolactone (GBL) in *Agrobacterium tumefaciens* C58 (Carlier *et al.*, 2004). The expression of the *attKLM* operon was activated in the presence of GBL and *Agrobacterium tumefaciens* C58 did not accumulate 3OC8HSL as it did when the *attKLM* operon was not induced (growing on mannitol). The *attKLM* operon-induced *Agrobacterium tumefaciens* C58 also became able to inactivate exogenous C6HSL and 3OC8HSL. Thus, it demonstrated a genetic link between the GBL degradation pathway and acyl-HSL-mediated quorum-sensing system.

Arthrobacter strain IBN110, a soil isolate, was found to be able to degrade various acyl-HSLs (Park *et al.*, 2003). Using a whole-cell assay, the strain degraded C4HSL, C6HSL, 3OC6HSL, C8HSL, C10HSL, 3OC12HSL, with C8HSL to be the most effectively degraded. The acyl-HSL-degrading lactonase was identified and designated AhlD. By using HPLC and ESI-MS, the digestion product of C6HSL by AhlD was confirmed to be hexanoyl-homoserine. Acyl-HSL-degrading activities were also observed in *Bacillus stearothermophilus* KCTC3067, and *Klebsiella pneumoniae* KCTC2241 in which another acyl-HSL lactonase of AhlK was identified.

C. Biochemical Degradation by Acyl-HSL Acylases

Through another distinctive mechanism, the amide bond of acyl-HSLs can be cleaved by acyl-HSL acylases (Fig. 1A), first discovered in a soil isolate *Variovorax paradoxus* VAI-C that utilizes acyl-HSLs as sole source of energy and nitrogen, releases HSL as a product of these reactions, and metabolizes the acyl moiety as energy substrate (Leadbetter and Greenberg, 2000). *Variovorax paradoxus* VAI-C was

able to grow on 3OC6HSL as sole source of carbon, energy, and nitrogen, although its growth rate was largely increased when NH_4Cl was amended (ca. five times faster). The bacterium could grow on the full spectrum of saturated acyl-HSLs tested as sole energy source. Furthermore, a linear relationship was observed between the molar growth yields on those molecules (C4HSL, C6HSL, C8HSL, C10HSL, and C12HSL) and their acyl chain length. HSL was detected in the culture fluid by quantitative amino acid analysis as a major degradation product that could serve as nitrogen source for growth. By using radiolabeled C4HSL (^{14}C at the ring carbon position one) as energy source, 56% of recovered radiolabel was in $^{14}CO_2$, thus demonstrating subsequent cleavage of the ring. Moreover, when the radiolabeled C4HSL was used as nitrogen source, more complete utilization of the molecule was observed, that is, 95% of recovered radiolabel was in $^{14}CO_2$.

The first gene encoding a protein with acyl-HSL acylase activity, designated *aii*D, was later identified in a *Ralstonia* strain XJ12B, isolated from a mixed-species biofilm (Lin *et al.*, 2003). The bacterium was capable of growing on C4HSL and 3OC12HSL as sole energy source, while purified AiiD exhibited significantly less inactivation on 3OC6HSL than other long-chain acyl-HSLs tested (i.e., 3OC8HSL, 3OC10HSL, and 3OC12HSL). By using HPLC and ESI-MS, as well as 5-dimethylamino-1-naphthalensesulphonyl chloride (dansyl chloride) derivation to increase the hydrophobicity of amino acids for HPLC separation, HSL (dansylated) was detected as the major product of digested 3OC10HSL by purified AiiD, thus confirming AiiD to be an acyl-HSL acylase.

A homologue of the *aiiD* gene was identified in *Pseudomonas aeruginosa* PAO1, *pvdQ*, and was shown to be an acyl-HSL acylase (Huang *et al.*, 2003). *Pseudomonas aeruginosa* PAO1 and a soil isolate *Pseudomonas* strain PAI-A were found capable of degrading long-chain ($\geq$8 carbons) acyl-HSLs and utilizing them as sole source of carbon and energy (Huang *et al.*, 2003). By using liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (LC/APCI-MS), HSL was directly detected as acyl-HSL degradation product from cell-free culture supernatants. *Escherichia coli* cells expressing *pvdQ* degraded acyl-HSLs and released HSL, which was also specific toward long-chain ($\geq$8 carbons) acyl-HSLs. However, although *pvdQ* was found to be sufficient, it was not necessary for acyl-HSL degradation in this species, as *pvdQ* knockout mutants remained capable of degrading 3OC12HSL. The second acyl-HSL acylase has been identified in *Pseudomonas aeruginosa.* Designated QuiP, it is the product of *Pseudomonas aeruginosa* gene PA1032 (Huang *et al.*, 2006). Acyl-HSL degradation and stoichiometric amounts of HSL accumulation

were determined by LC/APCI-MS from culture supernatants of *Escherichia coli* expressing recombinant *quiP*. Not surprisingly, QuiP has a specificity for long-chain (≥7 carbons) acyl-HSLs that is similar to PvdQ.

An acyl-HSL acylase designated AhlM has been identified in a *Streptomyces* strain M664 (Park *et al.*, 2005). By using HPLC and ESI-MS, as well as *o*-phthaldialdehyde (OPA) derivation for HPLC separation, HSL (OPA derivative) was detected as the digestion product of C10HSL by purified AhlM. The enzyme exhibited much more effective degrading activity with long-chain acyl-HSLs (≥8 carbons), that is, substantial decrease for C6HSL and 3OC6HSL and virtually no activity for C4HSL. Moreover, AhlM was more active against C8HSL than 3OC8HSL that has an oxo-substitution at the third carbon position.

Rhodococcus erythropolis W2, an isolate from a tobacco rhizosphere (Uroz *et al.*, 2003), was shown to degrade acyl-HSLs by both acylase and oxidoreductase (see below) activities (Uroz *et al.*, 2005). The bacterium was capable of utilizing diverse acyl-HSLs as sole carbon and energy source with a preference for short-chain acyl-HSLs (Uroz *et al.*, 2005). By using HPLC and LC-MS, as well as dansyl chloride derivation for HPLC separation, HSL (dansylated) was detected as the product from any of the 3OC10HSL, 3OC6HSL, or 3OHC10HSL being degraded by crude cell extracts of *Rhodococcus erythropolis* W2, thus confirming existence of acyl-HSL acylase activity. There were also isolates identified belonging to the genera *Pseudomonas, Comamonas, Variovorax*, and *Rhodococcus* able to degrade acyl-HSLs (Uroz *et al.*, 2003).

D. Biochemical Degradation by Acyl-HSL Oxidoreductase

Besides the acyl-HSL acylase activities described above, *Rhodococcus erythropolis* W2 has been shown to possess a novel oxidoreductase activity (Uroz *et al.*, 2005).

Resting *Rhodococcus erythropolis* W2 whole cells in phosphate buffer saline (PBS) much more efficiently degraded acyl-HSLs with the oxo-substitution at the third carbon position of the side chain than those without. By using HPLC and LC-MS, 3-oxo-substituted long-chain (≥8 carbons) acyl-HSLs were detected to be initially converted to their corresponding 3-hydroxy derivatives by whole W2 cells, but not the crude cell extracts, thus demonstrating a novel oxidoreductase activity associated with whole cells. Although 3OC6HSL was not reduced to the corresponding 3-hydroxy counterpart, it was still almost completely utilized by *Rhodococcus erythropolis* W2 growing cells by acyl-HSL acylase as described above.

Interestingly, the oxidoreductase activity is not specific for acyl-HSLs. It also reduced 3-oxo-6-phenylhexanoyl-homoserine lactone (3O6PhC6HSL) and 3-oxododecanamide (3OC12NH2) to their corresponding hydroxy counterparts. It is not stereospecific either since both D- and L-isomers of 3OC12HSL were converted to 3OHC12HSL.

E. Inactivation Reactions Involving Oxidized Halogen Antimicrobials

Oxidized halogens are widely used for microbial control in natural and industrial systems, which have been found capable of rapidly inactivating acyl-HSLs with oxo-substitution at the third carbon of the side chain (Brochardt *et al.*, 2001; Michels *et al.*, 2000).

The nonenzymatic degradation pathway of acyl-HSLs by oxidizing hypochlorite and stabilized hypobromite has been illustrated (Michels *et al.*, 2000). Both LC/DAD (photodiode array UV spectroscopy) and LC/APCI-MS results showed that when 3OC6HSL was oxidized by stabilized hypobromite at pH 8, it was rapidly dibrominated at the α-carbon position (i.e., between the two carbonyl groups of the side chain), and the last four carbons (i.e., from the β-carbon which is next to the halogenated carbon) of the side chain were shorn off, thus being converted to the α,α-dibromoethanoyl HSL (DBEHL) and releasing a 4-carbon butyric acid. The following step, which was the same as pH-hydrolysis, DBEHL was slowly hydrolyzed to the corresponding open-ring form, that is, α,α-dibromoethanoyl-2-(4-hydroxy)butanoic acid (hyd-DBEHL). Furthermore, at acidic pHs (pH 6 and 3) to slow the reaction rate, mono and dibrominated compounds, α-momobromo-3OC6HSL and α,α-dibromo-3OC6HSL, were detected as precursors to DEBHL, thus demonstrating a stepwise modification of 3OC6HSL. Same results were obtained for hypochlorite as well. However, such reactions were only observed with oxo-substituted acyl-HSLs at the third carbon of the acyl chain, as compared to straight-chain acyl-HSLs.

Hypochlorous and hypobromous acids (HOCl and HOBr) were found to rapidly react with acyl-HSLs with a 3-oxo group and eliminate the molecules' function as quorum-sensing signals, while straight-chain acyl-HSLs were not affected (Brochardt *et al.*, 2001). Furthermore, deactivation of 3OC6HSL was observed by HOBr in the marine alga *Laminaria digitata*. The formation of HOBr is catalyzed by bromoperoxidase in the presence of bromide and hydrogen peroxide (H_2O_2), which are found in seawater and produced by *Laminaria digitata*, respectively. Interestingly, the reaction between 3OC6HSL and stabilized

hypobromite in a *Pseudomonas aeruginosa* biofilm medium occurred. DEBHL and hyd-DBEHL were detected as described above, despite the much higher level of biofilm components.

F. Inactivation by Eukaryotes

1. *Inactivation by Mammalian Sera or Tissues*

All three PON genes, *PON1, PON2*, and *PON3*, are highly conserved in mammalian animals; PONs or PON-like proteins can be found in all animal species (Draganov *et al.*, 2000, 2005). PON1 is synthesized in the liver and secreted into the blood; PON3, which is also expressed mostly in the liver and some in the kidney, is found ca. 100 times less than PON1 in the serum, whereas PON2 is cell-associated and expressed in many tissues, including brain, liver, kidney, and testis, but not found in serum (Draganov and La Du, 2004; Draganov *et al.*, 2000, 2005; Ng *et al.*, 2001; Reddy *et al.*, 2001). Recently discovered acyl-HSL inactivation activities by mammalian sera or tissues are most likely caused by PON enzymes, based on their enzymatic characteristics, for example Ca^{2+}-dependent and sensitive to ethylenediaminetetraacetic acid (EDTA) (Chun *et al.*, 2004; Draganov *et al.*, 2005; Ozer *et al.*, 2005; Yang *et al.*, 2005).

Human airway epithelia has been shown capable of degrading several acyl-HSLs tested, including C6HSL, 3OC12HSL, and C12HSL, but not C4HSL or 3OC6HSL, and such activity is cell membrane-associated (Chun *et al.*, 2004). Furthermore, diverse mammalian cells from different animals and/or organs were shown possessing variable acyl-HSL-inactivating capacity on 3OC12HSL but not C4HSL, including cell lines from human colon carcinoma, human bronchoalveolar carcinoma, human cervix, human kidney, human lung fibroblasts cultures, monkey kidney, and canine kidney (with a decreasing capability), but not Chinese hamster ovary (CHO). Later on, by using proton-NMR, HPLC, and ESI-MS, such activity was shown to be lactonase activity, that is, hydrolyzing the lactone ring of 3OC12HSL to the corresponding 3-oxododecanoyl homoserine (Ozer *et al.*, 2005).

All three purified human PONs (PON1, PON2, and PON3), which were extracted from recombinant commercial *Trichoplusia ni* High Five insect cells with optimized procedures, have been shown to be lactonases/lactonizing enzymes through enzymatic studies and capable of hydrolyzing acyl-HSLs (Draganov *et al.*, 2005). All three purified PON enzymes degraded the DL-acyl-HSLs tested (except PON3 on

3OC6HSL), that is, 3OC6HSL, C7HSL, C12HSL, and C14HSL which were especially much more effectively hydrolyzed by PON2. Furthermore, the hydrolysis was found to be stereoselective since only half of the DL-acyl-HSL pool was hydrolyzed whereas all of the L-3OC6HSL was completely hydrolyzed by PON2. In addition, all such activities were fully inhibited in the presence of EDTA.

The rabbit serum has been shown to be able to degrade a full range of acyl-HSLs from C4HSL to 3OC12HSL, with a preference to long-chain acyl-HSLs (Yang *et al.*, 2005). HPLC and ESI-MS results showed lactonase-like activity of the rabbit serum. Likewise, such inactivation could be completely inhibited by EDTA and fully rescued by adding Ca^{2+}, which resembles those of PONs. Furthermore, mammalian sera samples from mouse, rabbit, horse, goat, human, and bovine, but not chicken or fish, demonstrated strong yet comparable inactivation activity on 3OC12HSL, with mouse and rabbit showing relatively higher activities. Such activity could be inhibited by EDTA as well. *PON1*, *PON2*, and *PON3* genes from mouse were cloned and expressed in CHO cell line, whose acyl-HSL inactivation activities were significantly increased to comparable levels despite the difference of the three PONs, thus confirming PONs could degrade acyl-HSLs.

Human and mouse sera have also been shown capable of rapidly inactivating 3OC12HSL, which can be largely inhibited by EDTA as well (Ozer *et al.*, 2005). HPLC and ESI-MS results further confirmed a lactone ring-hydrolyzing activity. Interestingly, mice serum lacking *Pon1* almost completely lost 3OC12HSL-degrading ability, while adding back purified human PON1 was sufficient to rescue such ability. Therefore, PON1 is most likely responsible for the acyl-HSL inactivation in mouse serum. Furthermore, CHO cells transfected with recombinant adenoviruses expressing three human PON enzymes all very effectively degraded 3OC12HSL, with PON2 the most active.

2. *Inactivation by Plants*

Legume *Lotus corniculatus* plantlets have been shown capable of degrading several acyl-HSLs, that is, C6HSL, 3OC6HSL, 3OC8HSL, and 3OC10HSL, whereas C6HSL appeared to be stable in the gnotobiotic root system of wheat and corn (Delalande *et al.*, 2005). Both bioassay and HPLC results demonstrated C6HSL disappearance from the medium. The acyl-HSL inactivation ability of the *Lotus* crude extracts was lost on boiling, thus supporting the view that it was due to enzymatic activities. The underlining mechanisms are not clear.

IV. Specificity of Acyl-HSL-Degrading Enzymes

Several bacteria have acyl-HSL-degrading enzymes that have activity against a broad range of acyl-HSLs, whereas others are more specific. *Variovorax, Ralstonia*, and *Bacillus* sp. contain enzymes that degrade a variety of both long- and short-chain length acyl-HSLs (Dong *et al.*, 2000; Leadbetter and Greenberg, 2000; Lin *et al.*, 2003; Park *et al.*, 2003), whereas *Streptomyces* and *Pseudomonas* seem to have a preference for long-chain acyl-HSLs (Huang *et al.*, 2003, 2006; Park *et al.*, 2005). Some bacteria have preferences for substitution at the third carbon position: *Rhodobacter* sp. degraded C6HSL better than 3OC6HSL (Uroz *et al.*, 2005), whereas the converse was true of *Variovorax* (Leadbetter and Greenberg, 2000).

Given that some acyl-HSL synthases produce mixture of acyl-HSLs (Fuqua *et al.*, 2001; Gonzalez and Marketon, 2003; Marketon and Gonzalez, 2002), it is interesting to speculate that enzymes that degrade acyl-HSLs could have a wider specificity for degradation to ensure that acyl-HSLs that could potentially be bioactive are degraded when their functions are not needed. In bacteria that produce acyl-HSL signals and use the degradation mechanism to regulate quorum sensing, the degrading enzymes might have widened specificity because there is not selection against this or because relaxed specificity selected the ability to degrade noncognate acyl-HSLs that might have activity. Acyl-HSL-degrading enzymes with broad specificity could enable non-acyl-HSL producing strains to degrade a wide range of signaling molecules produced by other organisms.

Acyl-HSL acylases identified have been shown to have homology to other acylases and the N-terminal nuclease hydrolase family of proteins (Lin *et al.*, 2003). Many acyl-HSL acylase enzymes are similar to penicillin amidase proteins, but they differ in the range of substrates they can degrade. AhlM from *Streptomyces* was able to degrade penicillin G by deacetylation indicating that this enzyme has broader specificity (Park *et al.*, 2005), whereas the *Ralstonia* sp. acylase does not degrade penicillin and many penicillin acylases do not degrade acyl-HSL. Lactonases identified have a range of acyl-HSL substrate specificities, but other functions of these enzymes have not been identified (Dong and Zhang, 2005). *In vitro* protein studies of the substrate kinetics for these enzymes may suggest what natural substrates these enzymes could degrade in nature.

Biologically synthesized acyl-HSLs are presumed to be in the L-form (Watson *et al.*, 2002). Consistently, bioassay revealed that L-isomers were essential as the autoinducers in quorum sensing, whereas no effect

was seen with D-isomers of acyl-HSLs, which were neither agonists nor antagonists (Ikeda *et al.*, 2001). Another way of acyl-HSL inactivation could be conversion to the D-form (Roche *et al.*, 2004), which is less active in *Erwinia carotovora* quorum sensing (Bainton *et al.*, 1992). Several acyl-HSL-degrading enzymes identified are not stereospecific for acyl-HSLs. *Pseudomonas aeruginosa* PAO1 degrades both D- and L-forms of the long-chain acyl-HSL, that is, C10HSL (Huang *et al.*, 2003). *Rhodococcus erythropolis* strain W2 was not stereospecific either in that it converted D-3OC12HSL to 3OHC12HSL (Uroz *et al.*, 2005).

V. Acyl-HSL Stability in Natural Environments

It is not unreasonable to argue that acyl-HSLs must have restricted stability in natural environments since highly stable molecules would accumulate over time and constantly exist at inducing concentrations despite fluctuations in population density, thus loosing their function as signals of such. Therefore, investigations into acyl-HSL stability in natural environments appear to be truly essential to understand such cell–cell communications.

The first study documented on acyl-HSL degradation by natural microbial communities was performed by using radiolabeling approach, and indeed demonstrated rapid acyl-HSL-biodegrading activities (Wang and Leadbetter, 2005). ^{14}C-labeled acyl-HSLs were mineralized to $^{14}CO_2$ when amended to fresh soil samples at physiological concentrations in nongrowth buffer and mostly without lag. The acyl-HSL degradation rate under optimal conditions, 13.4 nmol hour^{-1} g soil^{-1}, is more than two orders of magnitude faster than theoretical pH-hydrolysis as described earlier. To outpace such rapid degradation, an acyl-HSL-producing bacterium would have to reach a population density of greater than 10^9 or 10^{10} cells g soil^{-1} in the species-rich soil communities, based on the results that acyl-HSL synthesis rates appear to be no more than 10^{-18} moles cell^{-1} hour^{-1}, that is, when grown optimally *in vitro* (Dong *et al.*, 2000; Huang *et al.*, 2003; Lin *et al.*, 2003; Reimmann *et al.*, 2002; Zhang *et al.*, 2002). Moreover, the degradation activity by the soil communities can consume acyl-HSLs to an extent of less than 20 pM, which is far below the threshold concentrations required by many known acyl-HSL-producing bacteria to elicit a quorum response *in vitro*, that is, from 5 nM to 2 μM (Fuqua *et al.*, 1995; Kaplan and Greenberg, 1985; Pearson *et al.*, 1995; von Bodman *et al.*, 1998; Whiteley *et al.*, 1999).

pHs, ranging from 5 to 9, are prevailing common in many environments. Although acyl-HSLs are subject to pH-hydrolysis, rapidly

decomposing to the corresponding acyl-homoserines at circumneutral and higher pHs, they are quite stable for weeks or months at acidic pHs $\leq$6; however, none of acyl-HSLs, acyl-homoserines, or HSL is known to accumulate in the environment. The many acyl-HSL-degrading activities described above serve to explain part of this concern; studies have also been conducted to investigate utilization of the metabolized intermediates of acyl-HSLs (Fig. 1).

Acyl-homoserines, which are the open-ring hydrolyzed products of acy-HSLs, are known to be rapidly utilized as sole energy and nitrogen sources by an *Arthrobacter* strain VAI-A (Flagan *et al.*, 2003); while HSL, which is the amide bond cleaved moiety of acyl-HSLs, can be utilized as a nitrogen source by *Variovorax paradoxus* VAI-C and *Arthrobacter* strain VAI-A (Flagan *et al.*, 2003; Leadbetter and Greenberg, 2000), and as an energy source by several *Arthrobacter* strains (HSL-1, HSL-2, and HSL-3) and a *Burkholderia* strain HSL-4 (Yang *et al.*, 2006). HSL lactonase activity has also been found in *Pseudomonas aeruginosa*, but it is not observed to be utilized as a growth nutrient (Huang *et al.*, 2003). More importantly, mutual beneficial effects were clearly demonstrated in defined cocultures of *Arthrobacter* strain VAI-A and *Variavorax* strain VAI-C using 3OC6HSL as sole carbon source (Flagan *et al.*, 2003). Not only was the growth rate of VAI-A markedly enhanced by using viable cell counts, but also the biomass yield of cocultures increased dramatically than that of either of the monocultures (i.e., 6.3- and 1.8-fold greater for VAI-A and VAI-C, respectively) and that of the sum of their individual yields (i.e., 1.4-fold greater) under identical conditions. When 3OC6HSL was utilized as sole carbon and nitrogen sources, the effects were even more superior; the growth rates of each strain significantly exceeded those of the monocultures, and the biomass yield results were similar to the above described. Similarly, when any of the four isolates (*Arthrobacter* strains HSL-1, HSL-2, HSL-3, and *Burkholderia* strain HSL-4) was cocultured with *Ralstonia mannitolytica* strain SDV (encoding an acyl-HSL acylase) with C10HSL as sole energy source, the growth yields remarkably increased yet to variable extent (i.e., 11–26% depending on the isolates) than that of the monoculture of SDV (Yang *et al.*, 2006). Furthermore, degradation of acyl-HSLs by soil communities exhibited an apparent K_m of 1.5 μM, which is ca. 1000-fold lower than that of a purified acyl-HSL lactonase from *Bacillus cereus* (Wang *et al.*, 2004), suggesting that the observed degradation activities of soils are not mainly accounted for by acyl-HSL-degrading organisms currently available in culture (Wang and Leadbetter, 2005).

On the other hand, soils are not well-mixed systems; bacteria typically grow as microcolonies in soils. Although little is known of the natural distribution of quorum-sensing bacteria or acyl-HSL-degrading bacteria in soils, there has been evidence that signal degradation could serve to insulate microbial aggregates (Molina *et al.*, 2003). By using *Chromobacterium violaceum* CV026 as an acyl-HSL biosensor, which produces the purple pigmented violacein, acyl-HSLs production and diffusion were confirmed (as the biosensor being bright purple) from the acyl-HSL-producing bacterium *Erwinia carotovora* 852, when the *Erwinia carotovora* strain was spotted 16–17 mm away from the *Chromobacterium violaceum* line on LB agar plates. However, no induction of violacein was observed when a recombinant strain *Pseudomonas fluorescens* P3, expressing the *aiiA* gene from *Bacillus* sp. A24 that encodes a lactonase, was spotted in between the *Erwinia carotovora* and the biosensor at a distance of 6–7 mm from the biosensor line, clearly demonstrating degradation of surrounding acyl-HSLs by the P3 strain. Slightly weaker degradation activity was also observed by the wild-type *Bacillus* sp. A24. Therefore, such insulation of microbial aggregates from extraneous signals could disrupt beneficial or deleterious cross talk between spatially separated microbial populations that might otherwise occur (Blosser and Gray, 2000; Lewenza *et al.*, 2002; McDougald *et al.*, 2003; Pierson *et al.*, 1998).

Acyl-HSL-dependent quorum sensing is significantly influenced by the local temperature and pH. Lower temperature increases the half-lives of acyl-HSLs (Yates *et al.*, 2002). One reason why many marine bacteria use acyl-HSLs as quorum-sensing signals despite the high pH of seawater (ca. pH 8) is probably due to the low water temperature. Such concern should also be taken into account when culturing bacteria. For instance, LB is commonly used with aeration in laboratories; however, alkalization tends to happen due to release of ammonia from degradation of peptides as carbon and energy sources. On the contrary, anaerobic metabolism lowers the pH by producing weak acids. For example, in natural environment, it has been observed that soft rot, caused by quorum-sensing pathogen *Erwinia carotovora*, occurs more readily with limiting oxygen, probably because low pH helps *Erwinia carotovora* to accumulate quorum-sensing signal (Perombelon and Kelman, 1980; Yates *et al.*, 2002).

Long-chain acyl-HSLs appear to have greater advantages over short-chain acyl-HSLs in the environments. The longer the acyl side chain, the more stable they are at high pHs; once hydrolyzed, they can reform the ring and regain biological activity at circumneutral or mild acidic

conditions (depending on the pK of the carboxyl group) (Yates *et al.*, 2002). However, given their hydrophobic properties, long-chain acyl-HSLs would be more difficult to diffuse through cells and may be rapidly partitioned into organic compounds so that their function as quorum-sensing signals would be in question. Moreover, many acyl-HSL-degrading bacteria or enzymes have a specific or preference toward long-chain acyl-HSLs (Huang *et al.*, 2003, 2006; Lin *et al.*, 2003; Park *et al.*, 2005). For example, *Pseudomonas aeruginosa* PAO1 only degrades acyl-HSLs with chain length $\geq$8 carbons (Huang *et al.*, 2003) and *Streptomyces* sp. M664 much more effectively degrades acyl-HSLs with chain length $\geq$8 carbons (Park *et al.*, 2005). Although that sounds paradoxical, it may be the reason why some bacteria employs both short- and long-chain acyl-HSLs, for example *Pseudomonas aeruginosa* which produces both C4HSL and 3OC12HSL to control many of the same virulence genes (Latifi *et al.*, 1996; Passador *et al.*, 1993; Pearson *et al.*, 1994; Winson *et al.*, 1995).

VI. Coevolution of Quorum-Sensing Bacteria with Hosts and Acyl-HSL-Degrading Bacteria

Both plants and animals have sophisticated mechanisms to defend pathogens (Dangl and Jones, 2001) and in return, many bacterial pathogens have evolved strategies to overcome host defense, such as the population density-dependent quorum-sensing system. The pathogens elicit their virulence genes when enough population is achieved to overwhelm the host's defense response. It is possible that such antagonistic coexistence could lead to complicated coevolution of the pathogens and hosts.

As diverse bacterial species have been characterized to use quorum sensing to gain advantages over other competitors, it is not surprising to see those many mechanisms that bacteria and eukaryotes have evolved to degrade acyl-HSLs and thus disrupt quorum-sensing systems, as demonstrated by pure laboratory cultures, defined cocultures, laboratory microcosms, and soil microbial communities (see Sections III and V; for reviews, see Dong and Zhang, 2005; Zhang, 2003; Zhang and Dong, 2004). Besides, several other organisms have been shown capable of inhibiting or interfering quorum sensing via different mechanisms. For example, halogenated furanones from the marine red alga *Delisea pulchra* disrupt acyl-HSL-mediated quorum-sensing responses (Givskov *et al.*, 1996; Manefield *et al.*, 1999, 2001, 2002; Rasmussen *et al.*, 2000). Triclosan, an inhibitor of the enoyl-acyl carrier protein (ACP), reduced aycl-HSL biosynthesis *in vitro* (Hoang and Schweizer, 1999; Liu *et al.*,

2002). Pea *Pisum sativum*, crown vetch *Coronilla varia*, and alga *Chlamydomonas reinhardtii* have been shown to exude substances that mimic bacterial acyl-HSLs and interfere with quorum sensing (Teplitski *et al.*, 2000, 2004).

During this process, the hosts may have again evolved more complex defense mechanisms. One example is that in response to infection of a pathogen *Erwinina carotovora*, host plants can increase the pH of the apoplastic fluid around infection area by activating a rapid proton influx into the plant cells and making the intercellular pH to ca. 8, thus remarkably enhancing acyl-HSL hydrolysis and weakening virulence responses of *Erwinia carotovora* (Baker *et al.*, 1986, 1990; Byers *et al.*, 2002; Nachin and Barras, 2000).

Microorganisms usually evolve resistance mechanisms to counter antimicrobials. A good therapeutic agent would have to be able to eliminate the target bacterial virulence response without inhibiting the physiology of the bacterium; halogenated furanones from *Delisea pulchra*, which can interfere with quorum sensing, have been observed to have no appreciable inhibitory effects on growth of pathogenic bacteria over many generations (Bauer and Teplitski, 2001). However, very likely acyl-HSL degraders, or transgenic plants expressing acyl-HSL-degrading bacterial enzymes, may promote generation of bacterial strains capable of inhibiting such acyl-HSL degradation or to be acyl-HSL-independent for expression of virulence factors. In fact, such acyl-HSL-independent strains of *Erwinia carotovora* subsp. *carotovora* have been readily isolated (Whitehead *et al.*, 2001).

VII. Conclusions

In the past 30 years, the synthesis of acyl-HSLs and their role in the context of quorum-sensing systems have been studied intensively. It is only in the past 6 years have we realized that acyl-HSL degradation is just as important to a functional quorum-sensing system as production. For microbes that degrade acyl-HSL signals but are not known to accumulate acyl-HSLs or have quorum-sensing systems, acyl-HSL degradation could provide a means to compete with acyl-HSL-producing, quorum-sensing neighbors in the environment. Wang and Leadbetter (2005) have shown that acyl-HSL degradation by microbial consortia can proceed at physiological concentrations and to the extent below what is sensed by known quorum-sensing bacteria, indicating that acyl-HSL degradation poses challenges to quorum-sensing bacteria in natural communities. Acyl-HSL-degrading microbes that use acyl-HSLs as either a sole carbon source or carbon and nitrogen source (Huang *et al.*, 2003;

Leadbetter and Greenberg, 2000; Lin *et al.*, 2003) could gain an additional advantage in soil environments where nutrients are limited.

Surveys of many environments from the rhizosphere to marine habitats have identified an increasing number of bacteria that produce acyl-HSLs (Cha *et al.*, 1998; D'Angelo-Picard *et al.*, 2005; Wagner-Dobler *et al.*, 2005). The possibility that acyl-HSL-mediated quorum sensing is widespread suggests that we may be seeing tip of the iceberg in terms of microbes and organisms with acyl-HSL-degrading potential. Therefore, we have much to learn about the ways in which degradation is used and how it influences quorum-sensing microbes and communities. As more bacterial–bacterial and bacterial–eukaryotic interactions are studied, we may get closer to an understanding of the complexity of community interactions with acyl-HSLs.

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Microbial Exoenzyme Production in Food

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I. Introduction

Enzymes are responsible for the catalysis of almost all biochemical reactions. For centuries, man has used naturally occurring bacteria, yeasts, and molds and the enzymes they produce to make bread, cheese, wine, and so on. Before 1950, little information was available on microbial enzymes. Intensive studies started with the isolation of proteases from *Aspergillus oryzae* and *Bacillus subtilis* (cited in Morihara, 1974). Today, an overwhelming wealth of research data is available and desirable attributes of enzymes, most of them isolated from microorganisms, are of commercial value (Sharma *et al.*, 2001). Approximately 200 microbial enzymes are used for a wide range of applications either in food industry to improve texture, nutritional values, and to generate typical flavors and aromas or are used in the detergent-producing industry (Table I).

Undesirable substantial product deteriorations in foods of animal origin, however, are also attributed to the effects of extracellular microbial proteases and lipases (Baltes, 2000; Belitz *et al.*, 2001; Braun, 2003; Fehlhaber, 1992; Kraus, 1961). Endogenous enzymes on the other hand will not cause severe changes and are of minor importance for food deteriorations (Kraus, 1960).

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TABLE I

EXAMPLES FOR ENZYME-MEDIATED PROCESSES

Industry	Enzyme	Function
Dairy	Rennet (protease)	Coagulant for cheese making
	Lactase	Production of lactase-free milk products
	Proteases and lipases	For example production of roquefort
	Amylases	Production of fermented products
Brewing	Proteases and amylases, cellulases, β-glucanases	Breakdown of proteins and starch, remove cloudiness of beer during storage
Baking	Proteases and amylases	Lower protein levels in flour, breakdown of starch
Meat	Proteases and amylases	Ripening processes (tenderizing)
Paper	Amylases	Degradation of starch to lower viscosity
Cleaning detergents	Proteases and amylases	Presoak conditions and dish washing

Examples for undesirable organoleptic changes caused by microbial proteases (P) or lipases (L) in different kinds of food of animal origin are listed on page 61:

About one-third of the world's food production is lost annually as a result of microbial spoilage (Lund *et al.*, 2000) and presents an economically significant problem for manufacturers, retailers, and consumers. Spoilage can be defined as any change which renders a food product unacceptable for human consumption (Hayes, 1985).

To predict the onset of spoilage or to calculate shelf life of food, the total viable count (TVC) or even the count of psychrotolerant organisms may be of only limited value as indices of enzymatic activity (Christen and Senica, 1987; Kroll and Klostermeyer, 1984; Mottar, 1981; Picard *et al.*, 1994). Both parameters do not deliver any information on the metabolic activities of the microorganisms. Newly processed food can contain a variety of microorganisms and depending on the environmental conditions in a given food, only a small fraction of the microorganisms is actually of importance for product spoilage. In context of fish spoilage, the term of specific spoilage organisms (SSO) was created by Dalgaard (1995), meaning the flora growing faster than the remaining seafood microflora, and eventually produce the metabolites responsible for off-flavors and sensory product rejection during storage at

• Meat and meat products		
Fresh meat	Problems in ripening processes	P → *Bacillus, Pseudomonas, Aeromonas* and *Clostridium* spp., Yeasts
	Fruity aroma	L → *Micrococcus* spp.
Tins	Container blowing, decay	P → *Bacillus* and *Clostridium* spp.
Sausages	Decay, bitterness	P → *Bacillus, Clostridium, Serratia* and *Pseudomonas* spp.
	Rancid taste	L → *Bacillus, Staphylococcus* and *Micrococcus* spp.
• Milk and milk products		
Raw milk	Breakdown of casein, gelation	P → *Aeromonas* and *Pseudomonas* spp.
	Rancidity	L → *Aeromonas* and *Pseudomonas* spp.
Pasteurized milk	Sweet curdling	P → *Bacillus cereus*
	Unclean and bitter off-flavor	P → *Clostridium, Bacillus, Aeromonas* and *Pseudomonas* spp.
	Fat degradation	L → *Aeromonas, Pseudomonas* and *Proteus* spp.
Cream	Bitter flavor	P → *Pseudomonas, Bacillus* and *Aeromonas* spp.
Butter	Putrefactive type of spoilage	P → *Pseudomonas* and *Bacillus* spp.
	Rancidity	L → *Aeromonas, Pseudomonas, Bacillus* spp., *Serratia marcescens*
Hard cheese	Late gas blowing effect	P → *Clostridium tyrobutyricum*
Cheese	Reduced rennet coagulation time	P → *Pseudomonas* spp.
	Rancidity	L → *Pseudomonas* spp.
• Fish and fish products		
Fresh fish	Low temperature deterioration	P → *Pseudomonas, Aeromonas, Proteus* and *Bacillus* spp.
	Rancidity	L → *Pseudomonas, Aeromonas, Bacillus, Serratia* and *Micrococcus* spp.
Smoked fish	Dry and humid decay	P → *Bacillus, Pseudomonas* and *Aeromonas* spp.
Tins	Container blowing and decay	P → *Bacillus* and *Clostridium* spp.
• Egg and egg products		
Egg	Red, green, black decay	P → *Proteus, Pseudomonas* and *Serratia* spp.
	Fruity taste	L → *Pseudomonas* and *Bacillus* spp.
Fluid egg	Liquefaction/agglutination of egg content	P → *Pseudomonas* and *Aeromonas* spp.

particular conditions of temperature, atmospheres, salt percentage, a_w, preservatives, and so on.

Nevertheless, according to Gram *et al.* (2002), also the number of SSO present is not a reliable direct predictor of the sensory quality of a food, for example Braun and Sutherland (2003) detected proteolytic activity and synthesis of lipases at temperatures of 2–6 °C when pseudomonads achieved numbers of only 10^4–10^5 cfu ml^{-1}. In contrast, Ellis *et al.* (2002) noted a correlation between spoilage of chicken meat at 20 °C and a TVC of 10^7 cfu cm^{-2}.

Even sensory signs of spoilage often correlate poorly with TVC (Kraus, 1960, 1961). Dainty *et al.* (1975) observed proteolysis only when numbers of 10^9 cfu g^{-1} had been reached. As cited by Fairbairn and Law (1987), a psychrotroph count of at least 5×10^6 cfu ml^{-1} is necessary before proteolysis and 10^7 cfu ml^{-1} before organoleptic changes are detectable.

In the next chapters, general aspects such as structure, function, and classification of enzymes will be described before own studies on the synthesis and activity of microbial lipases and proteases under the influence of different parameters will be discussed in more detailed.

II. Structure and Function of Exoenzymes

Enzymes are mostly globular proteins of varying size, which often contain cofactors or coenzymes to function properly (Baltes, 2000; Belitz *et al.*, 2001). These can be either inorganic (e.g., metal ions such as Fe^{2+}, Mg^{2+}, Cu^{2+}, and iron–sulfur clusters) or heme, biotin, FAD, NAD, or coenzyme as organic molecules (prosthetic groups).

The long chains of amino acids of the enzymes are held together by peptide bonds, looped and folded into secondary and tertiary or quaternary structures by disulfide bonds, hydrophobic interactions, and salt bridges in order to enable their functional property such as efficiency and specificity. Enzymes are specific as to the reactions they catalyze and the substrates that are involved in these reactions because of shape, complementary charge, hydrophilic, or hydrophobe character of enzyme and substrate.

In an enzyme-catalyzed reaction, the substrate fits exactly to the so-called active site (usually thought of as a pocket-shaped gap in the molecule) of the enzyme. This is often referred to as ''the lock and key'' model to form a short-lived enzyme–substrate (ES) complex. Then the substrate is converted into the product while attached to the enzyme, and finally the product is released. This mechanism can be shown as:

$$S + E \underset{k_2}{\overset{k_1}{\leftrightarrow}} ES \overset{k_3}{\rightarrow} P + E$$

S = Substrate, E = Enzyme, P = Product, k_1, k_2, k_3 = rate constants.

Since the enzyme is not consumed or changed by the chemical reaction, it can be used over and over to catalyze additional substrate molecules.

In 1913, Michaelis and Menten proposed a quantitative theory of enzyme kinetics which is still widely used. Since the substrate concentration at V_{max} (i.e., the state where enzyme active sites are saturated with substrate) cannot be measured exactly, enzymes must be characterized by the substrate concentration at which the rate of reaction is half its maximum. This substrate concentration is called the Michaelis–Menten constant (K_M) and each enzyme has a characteristic K_M for a given substrate.

The metabolic rate at constant substrate and effector concentration and temperature is proportional to the enzyme concentration. The rate of reaction is the concentration of substrate disappearing or product produced per unit time (mol liter^{-1} s^{-1}). The enzyme activity is a measure of quantity of enzyme present. It is expressed as moles converted per unit time (rate $\times$ reaction volume), the SI unit is the katal (1 katal = 1 mol s^{-1}). In practice enzyme activity is specified as enzyme unit (EU) = 1 μmol min^{-1}.

Enzymatic activity can be investigated by either of the following methods:

- determination of the decrease in concentration of the raw substrate over time
- measurement of increase in concentration of degradation products during the reaction catalyzed by the enzyme (Kalisz, 1988).

In order to make sensitive and specific measurements of the often small enzyme quantities, the catalytic activity of the enzyme is used. The majority of existing methods supplies very exact results as, for example:

- photometric measurements, the Hull test, the Prescott and Wills measurement, the Azocasein method for proteases,
- the pH-stat method (titration), the Wilhelmy plate- and the Stead method, spectrometric methods, or the Reflektoquant Lipasetest (Merck) for lipases.

Photometric extinction measurements, however, are usually accomplished without exception in liquid media, which require by the majority a special expenditure according to device. Due to relatively simple execution and its reliability the agar diffusion method in diverse

modifications is widely applied (Christen and Marshall, 1984; Griffiths *et al.*, 1981; Kouker and Jaeger, 1987; Marshall and Marstiller, 1981; Stepaniak *et al.*, 1987).

III. Classification of Enzymes

Enzymes are classified by their E.C. number assessed by International Union of Pure and Applied Chemistry (IUPAC) and International Union of Biochemistry (IUB) in 1992:

- E.C. 1. Oxidoreductases
- E.C. 2. Transferases
- E.C. 3. Hydrolases
- E.C. 4. Lyases
- E.C. 5. Isomerases
- E.C. 6. Ligases

The loss of desirable food characteristics and substantial product deviations are, as already mentioned, mainly due to microbial growth of spoilage organisms and to the effects of their synthesized extracellular enzymes such as lipases, proteases, carbohydrases, and oxidoreductases which are characterized below.

A. Lipases

Lipases as triacylglycerolester hydrolases (E.C. 3.1.1.3) belong to esterases and catalyze the cleavage of water-insoluble esters thus releasing free fatty acids. The reaction is reversible so the enzyme can catalyze esterification of glycerol to form mono-, di-, and triglycerides. The process of lipolysis is often followed by further oxidative degradation of the liberated fatty acids into alkanes, alkenes, alcohols, aldehydes, ketones, and furanic cycles which results in context of spoilage experienced as rancidity. Typical lipolytic organisms are strains of the genera *Pseudomonas*, *Serratia*, *Micrococcus*, *Staphylococcus*, *Alcaligenes*, *Brevibacterium*, *Brochothrix*, *Lactobacillus*, yeasts, and molds which can be detected by tributyrin-containing media.

B. Proteases

Proteolytic enzymes (E.C. 3.4.) hydrolyze peptide bonds and therefore lead to the disassembly of proteins. The breakdown into smaller peptide molecules and eventually into their constituent amino acids and further to ammonium, alcohol, carbon dioxide, amines, and

hydrosulfide leads either to desirable sensory properties or to undesirable bitterness, gelation, putrid flavor, and so on.

Proteases are divided into two broad categories on the basis of how they cleave the polypeptide chain: *Exoproteases* cleave amino acids from the end of a protein chain at either the N-terminus or the C-terminus. *Endoproteases*, in contrast, are able to cleave protein inside the protein. In general, the term exoproteases (exoenzymes) is also used as synonym for extracellular proteases (or enzymes and is also used in the next chapters) which are released by cells after their synthesis and found free in the surrounding medium. In contrast, endoproteases or intracellular enzymes are found inside cells. However, the distinction between these and membrane-bound enzymes is often difficult. Enzymes may be membrane bound in young cells and released as exoenzymes as the culture enters stationary phase or solubilized by relatively mild procedures, including washing the cells with water (Cercignani *et al.*, 1974).

Furthermore, these two groups are further subdivided on basis of the mechanism of action at the active site into serine, cysteine (thiol), aspartic (acid), and metalloproteases. Peptidases can also be classified after their pH-optima where they work fastest: acid, neutral, and alkaline proteases. Most proteases are small, ranging from 21,000 to 45,000 Da.

Important proteolytes, for example, *Pseudomonas*, *Shewanella*, *Aeromonas*, *Acinetobacter*, *Moraxella*, *Proteus*, *Corynebacterium*, *Lactobacillus*, *Bacillus*, *Clostridium*, yeasts, and molds can be detected using protein-containing media with additives of casein, meat protein, gelatine, and so on.

C. Carbohydrases

Carbohydrases (E.C. 3.2.) hydrolyze polymers made up of various types of six-carbon sugars as the backbone of the polymer. Besides positive effects like acidification in ripening processes in fermented products deteriorations such as aerosis, slime formation (dextrans/levanes) and metabolites such as butyric acid or acetic acids can be observed. Glycolysis can be evaluated by counting acid-forming bacteria on starch-containing media (e.g., china-blue lactose agar).

D. Oxidoreductases

All enzymes catalyzing oxidoreductions belong to the group of oxidoreductases (E.C. 1). The substrate oxidized is regarded as a hydrogen or electron donor. The classification is based on ''donor:acceptor

oxidoreductase.'' Dehydrogenases (reductases) of microorganisms often lead to discolorations (color loss) of food by producing leuco-compounds. Reductases also set free volatile amines such as trimethylamine in fish which can be considered as an initial sign of spoilage. Microbial cytochromoxidases, synthesized for example by *Lactobacillus* spp., produce hydrogen peroxides and are therefore responsible for discoloration of sausages.

IV. Enzyme Synthesis

In general, the synthesis of enzymes follows the schema of protein biosynthesis consisting of transcription and translation that convert the genetic information of the DNA into protein. Several species, for example *Pseudomonas* spp., release multiple lipases and proteases simultaneously (Fehlhaber, 1992; Koka and Weimer, 1999). Further, Picard *et al.* (1994) showed that strains vary in their proteolytic activity: one *Pseudomonas fluorescens* strain excreted proteases which exhibit very low activity, while two other extracellular *Pseudomonas fluorescens* proteases showed strong enzyme activity. In principal, not all enzymes will be excreted, some will remain intracellular or bound to the cell (Kohlmann *et al.*, 1991).

A. Regulation Mechanisms

Two main opinions exist concerning the *regulation* mechanisms of extracellular enzymes:

1. Abbas-Ali and Coleman (1977) described a regulatory mechanism based on competition at the transcriptional level with the result that during exponential growth negligible amounts of extracellular proteases are synthesized owing to competition within the limited pool of nucleotide precursors. At the beginning of postexponential phase the pool of these precursors for RNA synthesis increases, owing to the turnover of stable RNA that finally leads to an increase in synthesis. The ''Coleman model'' seems to be applicable for Gram-positive organisms (cited in Fairbairn and Law, 1986).
2. The Harder's induction–repression model from 1979 is most likely appropriate for Gram-negative bacteria and takes into consideration that the regulation is based on three major steps: induction, end-product repression, and/or catabolite repression.

Harder assumed that microorganisms produce a very low basal level of extracellular enzymes in the absence of inducers. When substrates for these enzymes are present in the environment, small amounts of

inducer compounds will be liberated by their action and in this way the microorganisms obtain a signal indicating the presence of a potentially useful biopolymer.

Under appropriate nutrient limitation, synthesis is induced but may be also sensitive to negative feedback inhibition by the end-products such as the amino acids serine or isoleucine (so-called end-product repression); or enzymes are only produced if favored substrates such as C- and N-sources are already hydrolyzed (=catabolite repression, i.e., a decrease in the activity of certain auxiliary catabolic enzymes when a surplus of an easy metabolizable substrate, e.g., glucose or metabolites produced from glucose, is available).

More recent studies showed that Gram-negative bacteria are capable of actively exporting proteins beyond their cell envelope by using a highly complex and specific export machinery. Export components common among Gram-negative bacteria can be recognized and grouped into at least four different pathways (referred to as types I–IV; Lory, 1992; Pugsley, 1993; Salmond and Reeves, 1993). *Pseudomonas aeruginosa* as an example is a relatively prolific exporter of hydrolytic enzymes (e.g., alkaline protease) and is now known to possess at least three of the four export pathways (e.g., types I–III; type I, Filloux *et al.*, 1990; Guzzo *et al.*, 1991; Salmond and Reeves, 1993; type II, Durand *et al.* 2003; Kagami *et al.*, 1998; type III, Yahr *et al.*, 1996).

B. Important Inducers and Inhibitors

The synthesis of extracellular proteases is enhanced in the presence of peptides and certain amino acids, for example glutamine, asparagines, alanine, and leucine in the case of *Pseudomonas* proteinases (Amrute and Corpe, 1978; Fairbairn and Law, 1987; Hellio *et al.*, 1993; McKellar, 1982). Bovine serum albumin and milk proteins (primarily α_{s1}-casein) were reported by McKellar (1982), Whooley *et al.* (1983), and Fairbairn and Law (1987) as significant inducers.

However, proteinases are easily repressed by metabolic carbon sources such as citrate, glucose, galactose (Fairbairn and Law, 1987; McKellar, 1982; Patel *et al.*, 1983), or by ETDA in the case of metalloproteases, DFP and PMSF for serine proteases (McKellar and Cholette, 1985).

Olive oil, thiamine, tributyrin, Tween, and so on stimulate the secretion of lipolytic enzymes, whereas EDTA is also known as inhibitor for lipases (Ghosh *et al.*, 1996; Sugihara *et al.*, 1991).

Further, the presence of anorganic compounds such as calcium chloride is required for the stability of proteinases. Extracellular protease

yield per cell was stimulated ninefold by the presence of calcium chloride and production began immediately at the onset of growth and continued throughout the logarithmic phase (Amrute and Corpe, 1978).

C. Enzyme and Substrate Concentration

As the enzyme or substrate concentration increases the rate of the catalyzed reaction increases linearly. At very high enzyme concentrations the substrate concentration may become rate-limiting; at higher substrate concentrations the enzyme molecules become saturated.

D. Bacterial Growth

Apart from modulation by the mentioned factors, enzyme production is related to bacterial growth in three possible ways (Borriss, 1981):

1. Bacterial growth and enzyme synthesis occur concurrently.
2. Growth and enzyme synthesis commence at the same time, but production of extracellular enzymes continues after growth ceases so that these enzymes are detectable in the growth medium during the stationary phase.
3. Enzyme synthesis is undetectable until the end of the exponential phase, with a maximum during the stationary phase.

One or both of latter two possibilities seem to be more likely.

Limitation of nutrients at the end of exponential phase is the causative mechanism suggested for enhanced enzyme production in the stationary phase (Borriss, 1981; Fairbairn and Law, 1987). Further, Driessen (1981) assumed that bacteria accumulate the proteinase toward the end of their log phase. In their studies, proteases of *Pseudomonas fluorescens* in milk could be measured at the end of growth phase when bacterial numbers exceeded 10^7 cfu ml^{-1}. When the number of *P. fluorescens* was increased artificially by addition of milk that had been spoiled, proteolysis occurred already at 5×10^5 cfu ml^{-1}.

E. Influence by Physical Environment

Other important influencing factors on growth of microorganisms (according to Mossel, 1971) and also on synthesis and activity of enzymes they produce in food are the following:

''Intrinsic factors'' (e.g., pH-value, water activity, redox potential, presence of antimicrobial substances as well as other physical, chemical, and structural composition of the food)
''Extrinsic factors'' (temperature, relative humidity, and so on) and
''Implicit factors'' (behavior of the microorganisms within food)

Extracellular enzyme synthesis by bacteria is in general influenced by the physical environment, for example for *Pseudomonas fluorescens*, optimal production is observed at pH 6.5–7.0 (Amrute and Corpe, 1978; Fairbairn and Law, 1986), while an optimal temperature from 17.5 to 30°C for protease and lipase synthesis was described by several other authors (Hellio *et al.*, 1993; Juffs, 1976; McKellar, 1982). It is hypothesized that psychrotrophic bacteria such as *Pseudomonas* spp. synthesize increased quantities of enzymes at low temperatures to compensate for decreased enzyme activity at such temperatures, in order to maintain their growth rate (Fairbairn and Law, 1986; Juffs *et al.*, 1968; Schmidt-Lorenz, 1972), although this is at the expense of cell number. Not much is known about water activity as an influencing factor on synthesis except of that extremes generally inactivate the excretion of enzymes. In that way, many microbial enzymes have been extensively purified and characterized in terms of their activity and stability profiles relative to pH, temperature, and impacts of metal ions and chelating agents.

Limited work has been done on the synthesis and activity of microbial enzymes as affected by the combined effect of intrinsic and extrinsic factors in food. When spoilage is considered it is important to examine interactions between microbial growth and enzyme activity. Therefore, in the EU-project FAIR CT98–4083 data were collected on growth of cocktails of broadly similar organisms in conjunction with measurements of enzyme production, to attempt to produce a generic aerobic ''worst case'' food spoilage model for these organisms, combining both these aspects, applicable to any chilled perishable food.

The main food spoilage organisms were categorized into six groups:

- Group P: Pseudomonads and related species (*Pseudomonas lundensis*, *Pseudomonas fragi*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Shewanella putrefaciens*, and *Acinetobacter* sp.; Braun and Sutherland, 2003)
- Group Y: Yeasts (*Yarrowia lipolytica, Zygosaccharomyces bailii*, and *Pichia anomala*; Braun and Sutherland, 2004a)
- Group E: Enterobacteriaceae (*Escherichia coli*, *Enterobacter agglomerans*, *Klebsiella oxytoca*, *K. pneumoniae*, *Proteus vulgaris*, and one *Aeromonas hydrophila* strain; Braun and Sutherland, 2005)

- Group BT: *Brochothrix thermosphacta* (three strains; Braun and Sutherland, 2004b)
- Group LAC: Lactic acid bacteria (*Lactobacillus sake*, *Lactobacillus plantarum*, *Lactobacillus brevis*, *Leuconostoc gelidum*, *Leuconostoc carnosum*, *Carnobacterium pisicola*, and *Carnobacterium divergens*)
- Group BAC: Bacilli (*Bacillus cereus*, *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus megaterium*; Braun and Sutherland, 2004c)

The multiple strain cocktails (10^3 cfu ml^{-1}) were inoculated into liquid media with a pH of 4.5–7.5 and a range of water activities (0.95–0.995) preadjusted to conditions representative of foods and stored for up to 6 weeks at temperatures between 2 and 20°C. At defined intervals microbial count numbers were assessed, and in addition enzyme synthesis (lipases) and activity (proteases) and organic metabolic compounds related to increases in numbers of microorganisms were measured.

A slightly modified agar diffusion method was favored to generate data related to enzyme activity of Group P, B, BT, BAC, and E. Casein agar (adjusted to the defined pH-values and water activities) was used to detect proteolytic enzyme activity. Aliquots of 0.4 ml of enzyme-containing, bacteria-free filtrate were placed into containers created with plastic rings placed on the solidifying agar and incubated at temperatures corresponding with the growth temperatures (Braun and Sutherland, 2003) for 7 days. The area (cm^2) of the digested agar region (measured daily) is proportional to the enzyme activity which was confirmed independently by additional research groups (Griffiths *et al.*, 1981; Kouker and Jaeger, 1987).

The measurement of synthesized lipases is based on the conversion of Br,Cl-indoxylcaprylate by lipases to a blue dye (Reflektoquant Lipase-test, Merck) (Braun *et al.*, 2002). The final blue product concentration is assessed reflectometrically (measuring range 10–400 μg $liter^{-1}$). Broth volumes of 0.25 ml were taken at the same time intervals as for proteases for this purpose.

Representative examples for 300 combinations of environmental conditions per enzyme show in the following figures the effect of temperature, water activity, and pH on the enzyme synthesis and activity, respectively. Importantly, very low concentrations of lipases and proteases might not be detected due to the limits of the assays.

Effect of temperature: Maximal protease synthesis/activity of Group BT and BAC occurred at storage temperatures of 15 and 20°C. The proteases of Group P and Y were detectable between 2 and 20°C,

proteases of Group E including *Aeromonas hydrophila* develop their optimum at lower temperatures (2–10°C) and consequently give us an idea about their potential for spoilage of chilled products. Within the group of BT slight proteolytic reactions were observed.

Group Y and BAC produce high amounts of lipases at 15 and 20°C, Group E at 4, 6, and 10°C. No significant amounts of BT lipases were measured. Figure 1 shows the synthesis of lipases of Group P at 4, 6, 10, and 20°C in combination with a_w 0.995 and pH 7.5 over a period of 32 days. A decrease in temperature caused a significant reduction in lipase production but surprisingly even temperatures of 6°C did not inhibit the enzyme excretion.

The effect of varying pH and water activities on lipases of the P-group at a constant temperature of 20°C is shown in Table II. It is obvious that either a reduced water activity (0.97 or below) or lower pH inhibits very strongly the synthesis.

Effect of a_w: In general (observed in all groups) highest activity/synthesis of proteases and lipases was detected at a_w 0.995. Any reduction in the amount of water is associated with a loss of enzyme activity or with a delayed synthesis. So water activity seemed to be a very important limiting factor on exoenzymes. The impact of water activity on *Bacillus* proteases is illustrated in Fig. 2.

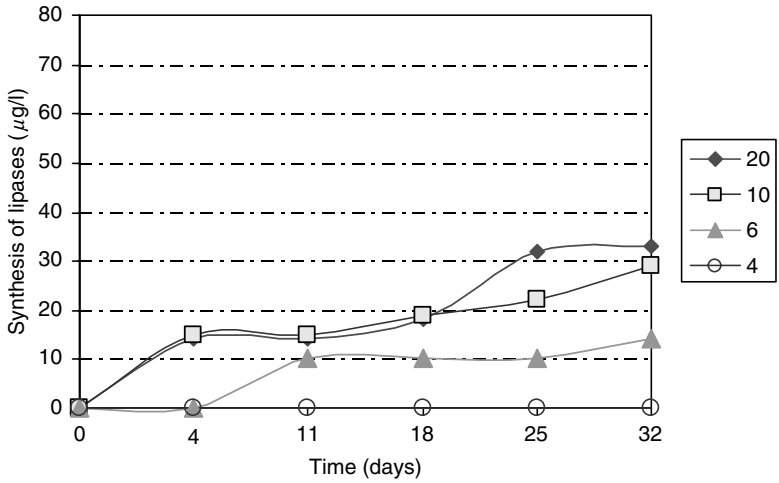

FIG. 1. Influence of temperature on the synthesis of lipases within the P-group at water activity of 0.995 and pH 7.5.

TABLE II

EFFECT OF pH AND WATER ACTIVITY ON LIPASE SYNTHESIS OF GROUP P AT 20°C

pH	a_w	0 day	4 days	11 days	18 days	25 days	32 days
7.5	0.995	0	14	14	18	32	33
7.5	0.98	0	12	12	15	15	16
7.5	0.97	0	10	10	12	14	11
7.5	0.96	0	Lo	Lo	Lo	Lo	Lo
7.5	0.95	0	Lo	Lo	Lo	Lo	Lo
6.5	0.995	0	10	13	15	23	26
6.5	0.98	0	Lo	Lo	10	10	11
6.5	0.97	0	Lo	Lo	Lo	Lo	Lo
6.5	0.96	0	Lo	Lo	Lo	Lo	Lo
6.5	0.95	0	Lo	Lo	Lo	Lo	Lo
5.5	0.995	0	10	11	16	23	25
5.5	0.98	0	13	13	14	14	14
5.5	0.97	0	Lo	Lo	Lo	Lo	Lo
5.5	0.96	0	Lo	Lo	Lo	Lo	Lo
5.5	0.95	0	Lo	Lo	Lo	Lo	Lo
4.5	0.995	0	11	11	12	16	16
4.5	0.98	0	10	10	11	10	10
4.5	0.97	0	Lo	Lo	Lo	Lo	Lo
4.5	0.96	0	Lo	Lo	Lo	Lo	Lo
4.5	0.95	0	Lo	Lo	Lo	Lo	Loz

Lo: <10 μg liter^{-1}.

At more unfavorable temperatures such as 10°C in combination with pH 7.5, proteolytic activity of the bacillus cocktail was seen when a_w ranged between 0.995 and 0.96, while no activity at the lower a_w 0.95 was observed. An acidic environment of pH 4.5 inhibited hydrolyses completely regardless of the combinations investigated.

Effect of pH: Proteolytic enzymes were most active and stable in the pH range 7.5–5.0 and their optimal reaction temperature was 20°C. Very acidic environment conditions as found in many fermented food products with a pH of 4.5 or below will inhibit proteolyses of the tested microorganisms completely.

Lipases were produced at all pH-values investigated, when combined with optimal water activities (a_w 0.995) or temperatures of

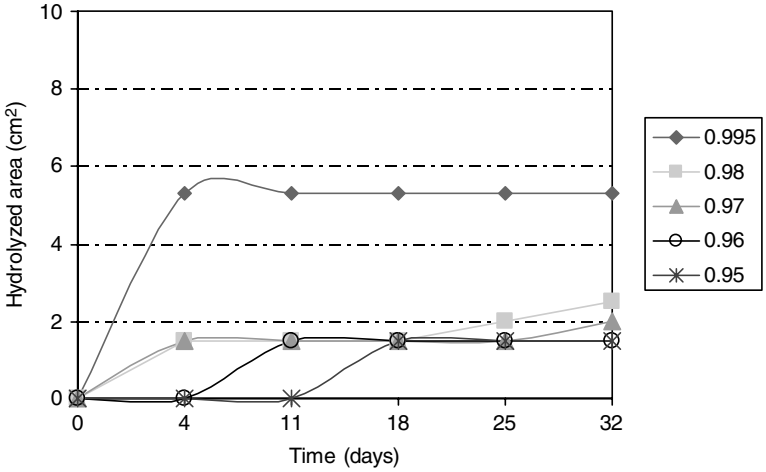

FIG. 2. Influence of water activity on the activity of *Bacillus* proteases at 20°C and pH 7.5.

15 and 20°C. Figure 3 illustrates a representative example for lipase synthesis of Group E at different pH-values, 20°C and a_w 0.995.

Nevertheless, it seems that production of bacterial enzymes under an unfavorable environment is slightly increasing in order to release nutrients for the growth or survival of the microorganisms. For example, at a_w of 0.995, in combination with pH 4.5 and 20°C, the bacillus cocktail secreted measurable lipase concentrations of 31 μg liter^{-1}, while at a_w 0.97 an increase to 41 μg liter^{-1} was observed, before the production finally ceased (no production at a_w 0.96).

The majority of enzyme synthesis was detectable during the late exponential and early stationary phase of bacterial growth when microbial numbers had reached the order of 10^7 cfu ml^{-1}, an observation that was also confirmed by Ellis *et al.* (2002). They had shown that at this level the main biochemical indicator of spoilage in chicken meat was onset of proteolysis. In exceptional cases, however, for example at 2°C, we observed that protease activity occurred even when microbial numbers were as low as 10^4–10^5 cfu ml^{-1}. These findings support similar results published by Mottar (1981), Kroll and Klostermeyer (1984), Christen and Senica (1987), and Picard *et al.* (1994). The amount produced or activity of the enzyme thereof did not correlate with the TVC of the microorganisms investigated at low temperatures (2–6°C) in particular.

The EU-project allowed us to develop growth models for all the pools investigated and valid for perishable foods. In most cases, a wider range

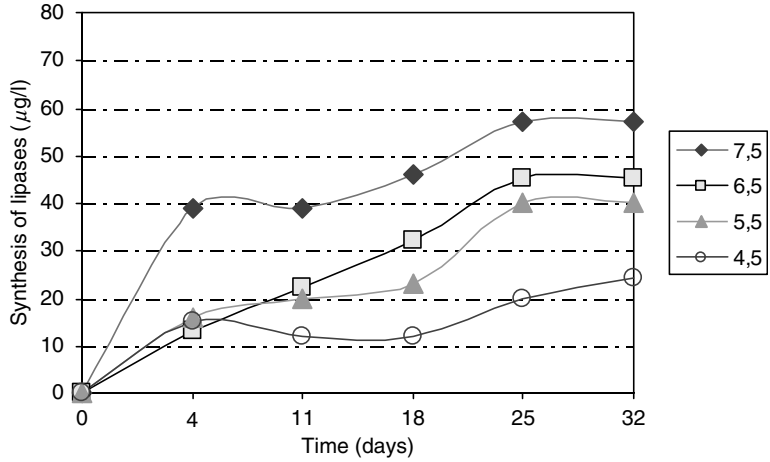

FIG. 3. Influence of the pH-value on the synthesis of Group E lipases at water activity of 0.995 and temperature of 20°C.

of environmental conditions compared to already existing models was included. The exponential phase of microbial growth was described with the model of Baranyi *et al.* (1993), difficulties were encountered in fitting it to the enzyme synthesis data. Consequently, it was not possible to produce a satisfactory model combining both microbial growth and bacterial enzyme production or activity. From these data it is obvious that alternative models need to be considered. However, models and enzymatic data were verified and validated in milk, cream, vegetables, beef, and poultry. For verification, the same cocktail of microorganisms used to construct the model was inoculated into sterile foods followed by incubation at a given temperature with regular enumeration of inoculated organisms and measurement of enzyme production or activity and increase with time. Afterward all data were validated, that is foods were allowed to spoil ''naturally'' at given temperatures and estimates were made of both TVC and numbers of specific groups of spoilage microorganisms and consequential increases enzyme synthesis or activity were recorded.

It is intended to develop a Web site that allows the access to spoilage models and data related to enzymatic synthesis and activity. This is part of the FAIR CT98-4083 project and should be accessible through the Web site of London Metropolitan University at www.londonmet.ac.uk.

In the context of validation and verification within the project, we measured lipase concentrations in the different products; comparable

quantitative data for hydrolytic enzyme concentrations (endogenous and microbial) in food do not exist. Therefore, additional investigations (Braun *et al.*, 2002) to estimate lipase concentrations and to evaluate their heat stability in selected food of animal origin (meat and meat products, fish and fish products, porcine, and turkey liver) were carried out using the Reflektoquant Lipasetest (Merck). Extremely high lipase concentrations were found in porcine and turkey liver (median values: 122 and 51 mg kg^{-1}, respectively) and in muscles of fat fish such as herring (915 $\mu g\ kg^{-1}$) or trout (1200 $\mu g\ kg^{-1}$). Pork contained 46 $\mu g\ kg^{-1}$, beef 86 $\mu g\ kg^{-1}$, and poultry up to 259 $\mu g\ kg^{-1}$. Heated sausage and hot smoked fish were mostly free of lipase, raw sausages, however, contained up to 283 $\mu g\ kg^{-1}$ lipases. Endogenous enzymes such as fish and meat lipases tested at varying conditions (50, 60, 70, and 80°C for 5 min) were not as heat-stable as investigated bacterial lipases which help to distinguish those from microbial enzymes. For example, a 5-min heating period at 50°C reduced the amount of fish lipases to 4–6%, of liver lipases to 10–12%, and of meat lipases to 37–87%. A temperature of 60°C leads to a fairly complete inhibition of fish and liver lipases, while meat lipases still showed 30–70% of their produced amount before they were finally inactivated at 70°C. Comparable investigations with *Pseudomonas* and *Staphylococcus* lipases showed 23–31% of the synthesized quantity at 70°C (heated for 5 min). However, further investigations including studies on proteases should be performed to show if a specific concentration can be linked to a certain best-before date.

V. Enzyme Activity

As explained for the synthesis of the enzymes in the chapters before, the enzymatic activity is also influenced by a number of parameters, which are described in parts below.

Temperatures (examples for lipases are listed in Table III) which are required for enzymatic hydrolyses can vary. In general, optimal enzyme activity can be expected in the 28–50°C range, with signs of protein denaturation above 45°C. Enzymes have a pH-optimum; maximal activities are observed between pH 4.5 and 8.0, for alkaline enzymes between pH 10 and 12. However, each individual enzyme has its own specific pH-optimum; examples for bacterial lipases are also listed in Table III. Water activity is another important factor limiting the hydrolysis by enzymes. The rate of enzymatic reaction in dried food with low water activity is limited by the rate at which the substrate diffuses to the enzyme. With regards to the structure of the catalytic center of enzymes,

TABLE III

EXAMPLES FOR TEMPERATURE- AND pH-OPTIMA OF SEVERAL BACTERIAL LIPASES

Bacteria	Temperature (°C)	pH-optima	References
Pseudomonas fluorescens	37	6.5	Law *et al.*, 1976
	40	8.5	Adams and Brawley, 1981
	22	7.0	Dring and Fox, 1983
	35	8.0	Fox and Stepaniak, 1983
	35	8.2	Stepaniak *et al.*, 1987
	23	8.0	Roussis *et al.*, 1988
	37	7.2	Bloquel, 1989
	50–55	8.0	Sztajer *et al.*, 1991
	45	7.5–8.5	Kumura *et al.*, 1993
	25	7.0	Abad *et al.*, 1993
	35	7.8	Marangoni, 1994
	45	8.5	Kojima and Shimizu, 2003
Pseudomonas aeruginosa	40	8.0–9.0	Finkelstein *et al.*, 1970
	50	8.5–9.0	Gilbert *et al.*, 1991
	30	6.5–7.5	Shabtai and Daya-Mishne, 1992
	55	10	Palmeros *et al.*, 1994
	45–50	9–10	Saeed *et al.*, 2005
Aeromonas hydrophila	30–40	7.0	Meyer, 1978
	35	–	Kalogridou-Vassiliadou, 1984
Staphylococcus aureus	40	8.0–8.5	Renshaw and Sou Clemente, 1967
	60	6.0	Muraoka *et al.*, 1982
	32–55	8.0	Halpin-Dohnalek and Marth, 1989
Bacillus subtilis	60	5.6–7.2	Sugihara *et al.*, 1991
	35	10.0	Lesuisse *et al.*, 1993
	65	8.2	Kim *et al.*, 1994
Serratia marcescens	–	8.75	Driessen and Stadhouders, 1974
	30–40	7.0	Meyer, 1978
	45	8.0	Matsumae and Shibatani, 1994
	37	8–9	Abdou, 2003

enzymatic activity is also influenced by effectors such as anorganic ions (Ca^{2+}, Mn^{2+}, Zn^{2+}, Mg^{2+}, and Fe^{2+}) or inhibitors, for example heavy metals, cysteine, or urea (Janssen *et al.*, 1994).

Enzymatic hydrolysis is reduced at unfavorable conditions. Nevertheless, residual activities, for example at suboptimal temperatures (see Table IV for several proteolytic organisms), are unexpectedly high.

With the exception of raw food, most products will be subjected to heat processes during manufacture. Even in these products enzymes are not completely inactivated. Examples for residual activities of heated proteases are: 92% for *Aeromonas hydrophila* heated at 56°C for 15 min (Nieto and Ellis, 1986), 86% for *Pseudomonas fluorescens* subjected to 45°C for 10 min (Malik and Mathur, 1984), 95% at 62°C for 30 min (Mitchell and Marshall, 1989), 8% at 120°C for 10 min (Patel *et al.* 1983), and 50% at 130°C for 25 s (Alichanidis and Andrews, 1977). Heat treatment of *Bacillus subtilis* at 70°C for 60 min resulted in 60% rest activity (Fujiwara and Masui, 1993) and for *Serratia marcescens* heated at 55°C for 60 min in 67.3% (Mitchell *et al.*, 1986).

TABLE IV

PROTEOLYTIC ACTIVITY AT TEMPERATURES BETWEEN 0 AND 25°C

Microorganism	Temperature (°C)	Remaining activity	References
Aeromonas hydrophila	4	35%	Alichanidis, 1988
Pseudomonas	25	25%	Adams *et al.*, 1975
fluorescens	4	33%	Alichanidis and Andrews, 1977
	20	72%	
	4	16%	Stepaniak *et al.*, 1982
	7	30%	
	5	High	Christen and Marshall, 1984
	4	19–27%	Stepaniak and Fox, 1985
	5	4%	Diermayr, 1986
	25	38–50%	Mitchell and Marshall, 1989
	5	Activ	
	0	24%	Hamamoto *et al.*, 1994
	10	38%	
	20	57%	

Some proteases and lipases of psychrotroph organisms such as pseudomonads appear to be susceptible to inactivation at relatively low temperatures (55°C for 10 min). This is referred to as low temperature inactivation by Barach *et al.* (1978) and other authors and an autolysis is thought to be the basis for this phenomenon.

However, many studies focused on finding the optimal conditions at which enzymes work fastest to use them as starter cultures. In order to prolong the shelf life of a product, it is necessary to know more about the behavior of enzymes in an unfavorable environment and how a combination of factors may influence the capabilities of hydrolases to cleave different substrates.

Our own investigations (funded by Deutsche Forschungsgemeinschaft, DFG) addressed this problem. To describe the enzymatic activity in food, proteases and lipases of 55 spoilage-causing bacterial strains of 13 different species were produced under optimal conditions (incubation of 72 h in fat- and protein-enriched nutrient broth) and measured by the modified agar diffusion test. Tween, tributyrin, gelatine, and casein were used a substrates. In order to investigate hydrolytic effects in heated products, the bacteria-free enzyme-containing filtrates were also tested after using different heating programs (60°C for 60 min, 65°C for 30 min, 71°C for 15 min, and 75°C for 5 min).

It can be concluded that simultaneous effects of pH-value, water activity, and temperature were the crucial factors for influencing enzyme activity and enzyme synthesis. Single environmental factors add up and can be compared with hurdles similar to the ''hurdle concept'' described by Leistner for the bacterial growth (Leistner and Gorris, 1995). This concept states that several inhibitory factors (hurdles), while individually unable to inhibit microorganisms, in combination will be effective as inhibitors. A classic example of applying the hurdle concept is the antibotulinal stability of certain shelf-stable processed cheese formulations. Combinations of moisture, total salt, and pH promote safe storage of these products at room temperature for extended time even though the individual factors alone would not support that practice. Turn to enzymatic activity specifically, reduced temperature, water activity, and pH-values also decrease or inhibit enzyme activity or enzyme synthesis of all enzymes. Nevertheless, surprisingly high activities were observed at chilling temperatures, for example for lipases and proteases of *Pseudomonas* spp., of *Aeromonas* and *Bacillus* strains. Heating of microbial enzymes results in a reduction of their activity but complete inhibition is not achieved. *Pseudomonas* spp.

particularly secret very stable proteolytic enzymes, observations that reported earlier by Cogan (1977), Law (1979), Griffiths *et al.* (1981), and Cousin (1982). *Aeromonas hydrophila* and *Serratia marcescens* too produce very heat-stable proteases which Nieto and Ellis (1986) or Mitchell *et al.* (1986) described for these strains.

Lipases of *Pseudomonas* spp., *Staphylococcus epidermidis*, and *Aeromonas hydrophila* have also to be assessed as heat resistant. Similar results presented Sztajer *et al.* (1991), Dring and Fox (1983) for pseudomonads and Kalogridou-Vassiliadou (1984) for *Aeromonas.*

Two linear mathematical models (predictive enzymology) using the method of Baranyi *et al.* (1993) are available which have been verified and validated in milk.

The ''short-time'' model helps to predict effects of both untreated and heated extracellular lipolytic and proteolytic enzymes of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Aeromonas caviae*, *Aeromonas hydrophila*, *Proteus mirabilis*, *Proteus vulgaris*, *Bacillus cereus*, *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Micrococcus luteus*, *Clostridium perfringens*, and *Serratia marcescens* over a period of 4 days (Braun and Fehlhaber, 2002) within the three-dimensional matrix of environmental conditions, including variation in temperature from 2 to 37°C, pH-values from 4.0 to 7.3, and water activities from 0.8 to 0.98. Examples of the short-term model for the expected proteolytic activity of *Pseudomonas fluorescens* at 10°C in combination with pH 7.3 and a_w 0.98 are shown in Fig. 4.

The ''long-time'' predictive model focuses on extracellular lipolytic enzymes synthesized by *Pseudomonas aeruginosa, Pseudomonas fluorescens*, *Aeromonas hydrophila*, *Staphylococcus aureus*, and *Serratia marcescens* within a temperature range from −2 to +7°C, pH-values from 4.0 to 6.3, and water activities from 0.95 to 0.995. It makes predictions possible over a period of 38 days (Braun and Fehlhaber, 2003).

Access to both models on enzymatic activities for selected spoilage organisms is provided by the Institute of Food Hygiene, Faculty of Veterinary Medicine, University of Leipzig (www.uni-leipzig.de/∼lmh).

VI. Conclusion and Future Prospects

There is an increasing number of risk factors originating from all parts of the food supply chain that present increasing challenges to control microbial spoilage. From the consumer perspective, there is a

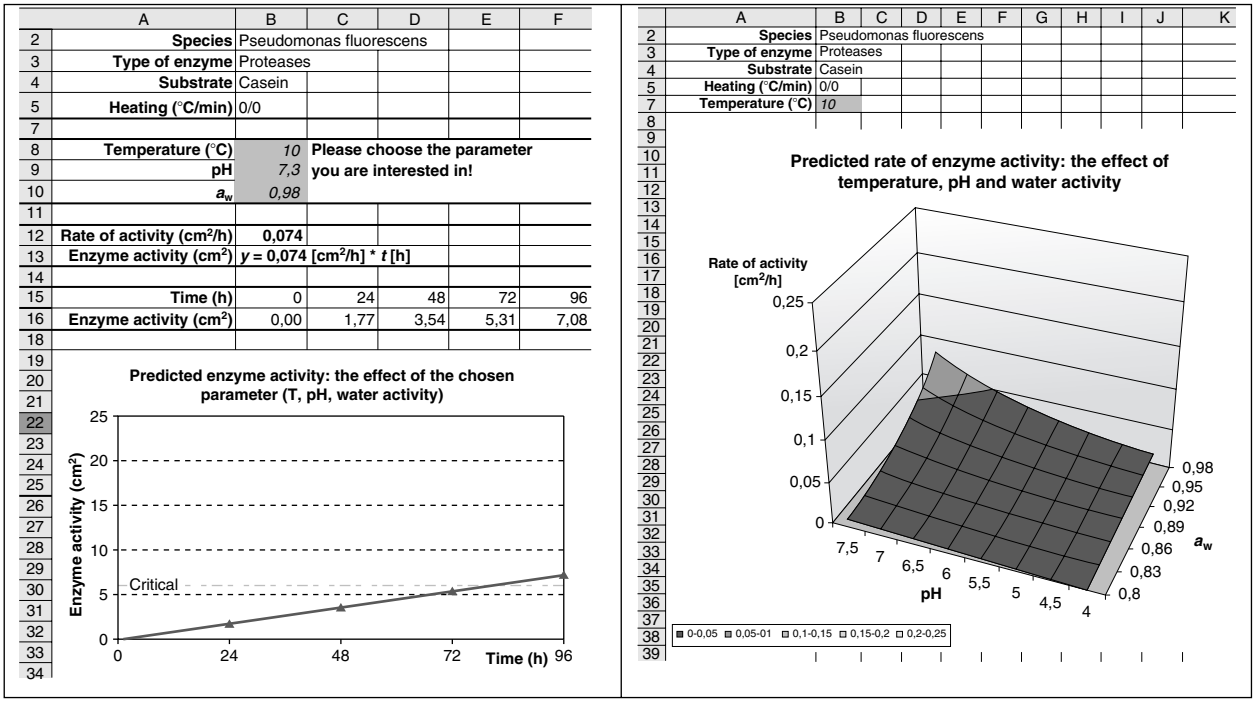

FIG. 4. Predicted activity of *Pseudomonas fluorescens* proteases at 10°C, pH 7.3, and a_w 0.98.

demand for minimally processed and organic foods, combined with the expectation of improved quality, guaranteed safety, long shelf life and competitive pricing. Together with the increasing variety of products the susceptibility to spoilage has enhanced, as has the diversity of spoilage organisms. As with microbial safety, it is proposed that the use of HACCP concepts, supported by programs such as Good Manufacturing Practice and Risk Assessment, provides means and chances to control spoilage (Blackburn, 2006).

Nevertheless, the underlying mechanisms of food spoilage are very complex and may be difficult to identify and are therefore still poorly understood (Huis in't Veld, 1996). It seems to be clear that a multidisciplinary approach is needed in the management of microbial spoilage. Understanding of microbial behavior and the ecology is fundamental for decision-making with regard to controlling food spoilage. All these data, generated within the EU and DFG project, are another helpful tool for the food industry and decision makers to predict shelf life or the onset of spoilage more precisely.

It is also necessary to acquire more knowledge on the mechanisms dictating which microorganisms become dominant in the microflora and implement it into control measures. Recent studies showed that selective advantages for a particular fraction microorganisms are due to, for example, production of siderophores by pseudomonads to utilize the limited amount of iron in the medium, or to metabiosis as a form of interdependency, or to the ability of pseudomonads and Enterobacteriaceae to coordinate expression of certain phenotypic traits (quorum sensing) through bacterial communication (Gram *et al.*, 2002).

Acknowledgments

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Biogenetic Diversity of Cyanobacterial Metabolites

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I. Introduction

Cyanobacteria (blue-green algae) are ancient prokaryotic photosynthetic organisms that have survived and flourished on the planet for over 2 billion years and produced the oxygen that enabled aerobic metabolism and all life-forms that depend on it (Dismukes *et al.*, 2001; Sergeev *et al.*, 2002; Thajuddin and Subramanian, 2005). Over this time, these robust organisms have adapted to survive in a multitude of microhabitats, from the polar caps to the equator, from land-based environments to ponds, lakes, and oceans (Fay, 1992; Sànchez-Baracaldo *et al.*, 2005; Tandeau de Marsac and Houmard, 1993;

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Thajuddin and Subramanian, 2005). Perhaps coupled to their tenacious survival, cyanobacteria produce a remarkable diversity of secondary metabolites, which may in part have contributed to their extraordinary success and successful colonization of the planet. Some metabolites act as natural photoprotectants (Sinha *et al.*, 1998), while others may provide protection from predators (Nagle and Paul, 1999), and others alter the stability (Mohamed *et al.*, 2005; Simonin *et al.*, 1996) or gas permeability (Murry and Wolk, 1989) of the thylakoid membrane.

While the role of many of these compounds in the function and survival of the producing organism has challenged researchers for many decades, cyanobacteria are perhaps best known for the toxins they produce, in particular those that affect mammals and humans. When linked to the fact that many cyanobacteria flourish in lakes, lagoons, and reservoirs which communities use as a supply of drinking water and recreational activities, this concern is easy to understand. Indeed, there have been numerous reviews examining the threat of cyanotoxins to the population and our way of life (Carmichael *et al.*, 2001; Chorus *et al.*, 2000; Dittmann and Wiegand, 2006; Falconer, 1999; Falconer and Humpage, 2005; Wiegand and Pflugmacher, 2005; Zurawell *et al.*, 2005) and on methods to detect and cope with the toxins (dos Anjos *et al.*, 2006; Hummert *et al.*, 2001; Lawton and Edwards, 2001; McElhiney and Lawton, 2005; Meriluoto, 1997; Msagati *et al.*, 2006; Spoof *et al.*, 2003; Svrcek and Smith, 2004). However, the ability of cyanobacteria to biosynthesize complex toxins underscores their innate ability to biosynthesize complex secondary metabolites in general, and more recently, the structural diversity of cyanobacterial metabolites has attracted the attention of scientists searching for new pharmaceutical leads (Sielaff *et al.*, 2006; Spolaore *et al.*, 2006). Today, cyanobacteria are recognized as an exciting resource of new compounds with pharmaceutical potential.

This interest in the range and structural diversity of cyanobacterial metabolites has expanded to examine the biosynthetic mechanisms required to produce such metabolites. From a chemotaxonomic perspective, arguably the most recognized and characteristic group of cyanobacterial metabolites are the peptides and depsipeptides (Section IV), all of which are unique to cyanobacteria, and many of which display a variety of biological activities. However, in reality, the cyanobacteria also produce an impressive number of polyketides (Section III) and alkaloids (Section V), many of which are also unique to cyanobacteria, having not been found in other organisms, whether land-based or aquatic. In recent years, this interest in the biosynthesis of cyanobacterial metabolites has grown to include the identification of the appropriate

biosynthetic genes (Dittmann *et al.*, 2001b; Ramaswamy *et al.*, 2006; Welker and von Doehren, 2006), and has even raised the possibility of engineering such genes to produce new ''semibiosynthetic'' compounds (Donadio and Sosio, 2003; Floss, 2006; Kantola *et al.*, 2003; Méndez and Salas, 2003; Rix *et al.*, 2002; Tsoi and Khosla, 1995; Walsh, 2002).

Many pioneering biosynthetic studies with cyanobacteria have revealed that almost all cyanobacterial peptides are produced by a complex enzyme system described as a nonribosomal peptide synthetase (NRPS) (Becker *et al.*, 2004; Hoffmann *et al.*, 2003; Tillett *et al.*, 2000). Such systems are capable of building peptides with protein or nonprotein amino acids, some of which are exotic in themselves, resulting in further structural diversity. Similar enzymes known as polyketide synthases (PKS) are equally capable of producing exotic molecules built from small acid units such as acetate or propionate (Hopwood, 1997). In very general terms, the NRPS and PKS enzymes operate in a similar fashion, namely the sequential addition of activated precursors to a nascent peptide or polyketide chain. As described in the next section, this has led to a remarkable interplay between these two systems, resulting in compounds with a mixed NRPS and PKS biosynthetic origin. Peptides derived in such a fashion are often referred to as hybrids or hybrid peptides. The NRPS and PKS complex of enzymes appear to retain notable flexibility in their design and architecture, resulting in many tens of compounds, all structurally unique. Indeed, when considered together, these biosynthetic processes account for the majority of all cyanobacterial secondary metabolites.

Section V describes the cyanobacterial alkaloids which generally result from the incorporation of nitrogenous compounds into polyketide-, isoprenoid-, or shikimate-derived precursors. Perhaps not surprisingly, cyanobacteria have been found to produce a remarkable suite of alkaloids many of which are unique to cyanobacteria (Gerwick *et al.*, 2001). In some cases, the biosynthetic genes and pathways leading to these complex alkaloids have been determined, and such studies underscore the complex biosynthetic processes invoked in their production and the extraordinary structural diversity that results. The final sections describe metabolites obtained from other biosynthetic pathways such as isoprenoids (Section VI) and shikimate-derived compounds (Section VII). To date, these have been less frequently encountered in cyanobacteria, which could merely be a consequence of a more intensive focus on peptidic compounds.

The purpose of this work is to present a comprehensive review of the biogenetic and chemical diversity of secondary metabolites produced by cyanobacteria. We have attempted to organize these compounds into

groups based on their biogenetic origin. Of course, the biosynthetic pathways of all cyanobacterial metabolites have not been elucidated, and some groupings have been created based on comparison with other cyanobacterial compounds, as well as secondary metabolites from other organisms including bacteria, fungi, and microalgae (e.g., dinoflagellates). We have not attempted to show every structure reported from cyanobacteria due to the large numbers that have been described. Instead, we have provided a sampling of the metabolites that have been described so far and which represent all of the major cyanobacterial structural types.

II. Major Biosynthetic Routes in Cyanobacteria

As in many other organisms, the majority of natural products isolated from cyanobacteria are made by some combination of two major biosynthetic paradigms: PKSs and NRPSs. These two biosynthetic processes are very similar to one another in three important ways that allow mixing and matching between them in a combinatorial fashion. First, in both biosynthetic models, simple activated subunits are covalently linked to form nascent linear chains. Second, these subunits can be varied or modified to provide even greater structural diversity in the final products. Third, both systems are structured in an assembly line fashion with separate domains performing specialized modifications on the nascent chain before passing it on to the next unit. Operating independently, both paradigms have provided a seemingly never-ending supply of natural products, and in combination, the interplay of these two biosynthetic systems has only served to increase this plethora of compounds. Furthermore, as the genetics of the process become even better understood, it will offer an opportunity to create an even larger library of products through molecular engineering techniques (Cane and Walsh, 1999; Schwarzer *et al.*, 2003; Weissman, 2004).

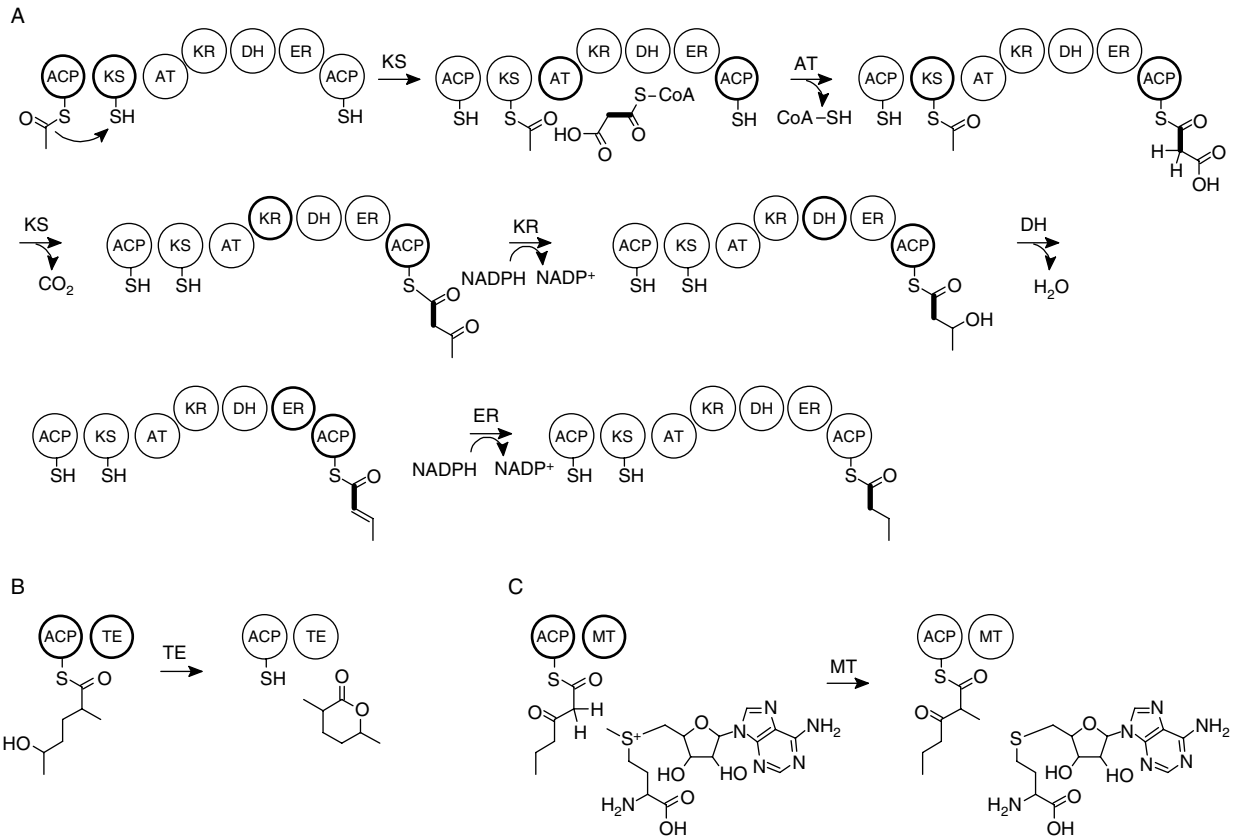
A. Polyketide Synthases

PKSs are similar to, and evolved from, fatty acid synthases (FASs), though they can vary considerably in their genetic architecture, whether they are iterative or processive (or modular), and whether intermediates are tethered to proteins or cofactors. There are many reviews of PKSs including those cited here (Hopwood, 1997; Shen, 2003; Staunton and Weissman, 2001), from which much of the following discussion is

drawn. In the interest of brevity, only the key aspects of polyketide synthesis that are most common or most general are described.

A typical PKS is made up of several genes. The genes usually contain multiple modules, each of which is responsible for the addition and subsequent processing of a single subunit in the nascent chain. The modules in turn are made up of multiple domains that each catalyzes one or a few chemical transformations on the subunit. These domains are well characterized and can often be identified easily within protein sequences.

The process begins when a starter unit, typically acetate but more complex carboxylic acids can be utilized, is loaded onto an acyl carrier protein (ACP) domain (Fig. 1A). Acyl intermediates are attached as thioesters of a phosphopantetheine cofactor that is covalently bound to the ACP. The cofactor is derived from coenzyme A (CoA) and is attached to an absolutely conserved serine residue. The priming reaction is followed by multiple steps of chain extension as illustrated in Fig. 1A. The nascent chain from the ACP of the previous module is transferred to an active site cysteine residue of a keto synthase (KS) domain. An extender unit, typically malonate (originally derived from acetate) in the form of malonyl-CoA, is selected and transferred to an ACP in the current module by an acyl transferase (AT) domain. As such, the AT domains act as gatekeepers for the entry of extension units into a particular location on the chain (Liou and Khosla, 2003). The activated malonyl ester undergoes Claisen condensation with the starter acetate unit to form a β-ketide unit. Next, this unit can undergo reduction to an alcohol by a keto reductase (KR) domain, which may be dehydrated to an olefin by a dehydratase (DH) and reduced to an aliphatic group by an enoyl reductase (ER). Often not all of the latter three enzymes are present and active, leading to different oxidation states of the extended chain. Usually, a nascent polyketide chain undergoes several extension reactions before release from the PKS by a thioesterase (TE) domain (Fig. 1B). The TE can act intramolecularly, leading to a cyclic product, or intermolecularly, leading to a linear product. Another commonly occurring domain in PKSs is the methyl transferase (MT) domain (Fig. 1C). MT domains catalyze the methylation of β-keto intermediates by nucleophilic attack on the methyl group of *S*-adenosylmethionine (SAM). The methyl groups of SAM are part of a pool of C_1 units that are easily enriched with stable isotopes, allowing researchers to distinguish whether pendant methyl groups are incorporated into polyketides by way of SAM or by way of methylmalonate. The former pathway is overwhelmingly dominant in cyanobacteria.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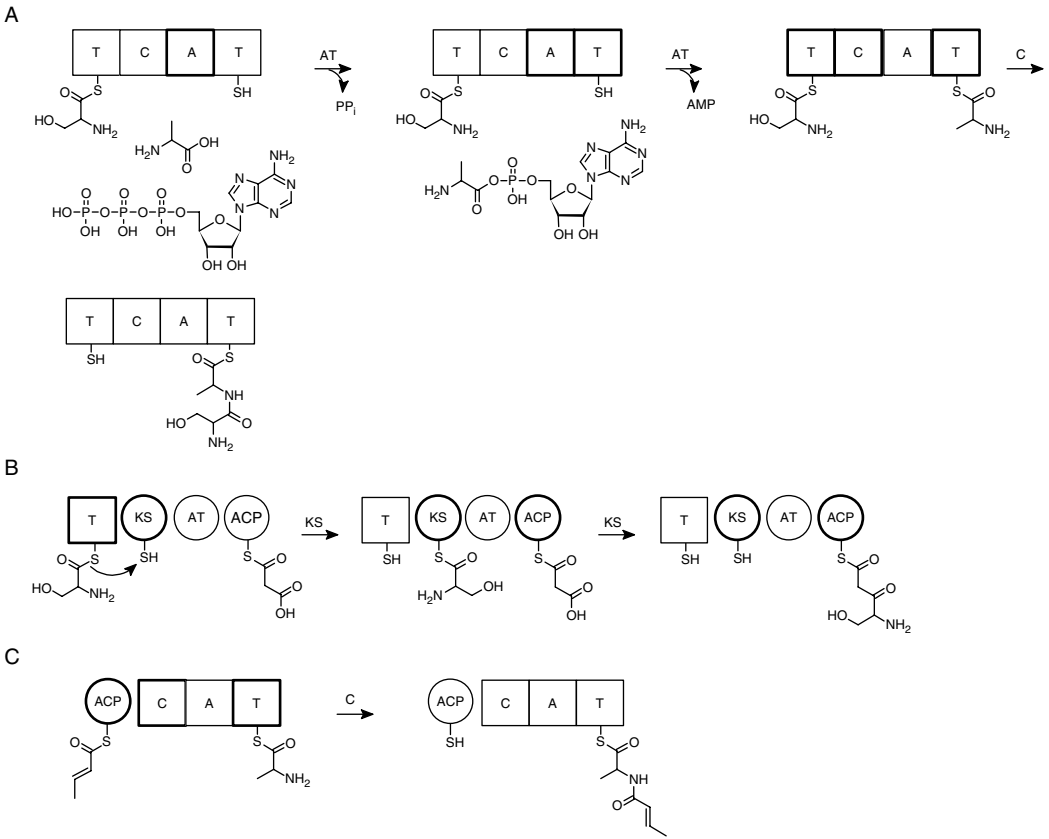
B. Nonribosomal Peptide Synthetases

NRPSs share the gross module/domain architecture of the PKSs (Cane and Walsh, 1999). Instead of ACPs, NRPSs contain thiolation (T) domains, also known as peptidyl carrier proteins (PCPs). T domains, like ACPs, also contain phosphopantetheine cofactors. A typical NRPS extension is shown in Fig. 2A. The gatekeepers of NRPS modules are the adenylation (A) domains, which activate carboxylate groups of the next amino acid selected to extend the chain. The activated amino acid forms a thioester bond to the T domain of the module. The condensation (C) domain then catalyzes the transfer of the nascent polypeptide chain to the amino group of the extender amino acid. Just as in PKSs, the amino acid sequence in peptides produced by NRPSs is determined by the sequence of modules in the synthase, in contrast to the mRNA-guided ribosomal production of polypeptides.

One of the features of NRPS and PKS modules that is often exploited by cyanobacteria is the ability to mix PKS modules with NRPS modules. This interplay of biosynthetic processes usually occurs within genes and leads to production of molecules with properties that differ greatly from what can be achieved with PKSs alone or with NRPSs alone. Figure 2B illustrates the case when an NRPS module is followed by a PKS module. The nascent polypeptide is translocated to the KS domain and subjected to Claisen condensation with the extender unit, just as in PKSs, resulting in formation of a new carbon–carbon bond. When a PKS module is followed by an NRPS module, the acyl chain forms an amide bond with the amino acid extender (Fig. 2C). Many examples of both configurations are present among cyanobacterial metabolites.

Fig. 1. Polyketide synthase reactions. This figure provides an overview of common reactions catalyzed by polyketide synthases. (A) A typical module from a polyketide synthase. Each circle represents a domain within a module. At each step the domains participating in a reaction are highlighted with a thick outline. A typical module will contain an ACP domain, a KS domain, an AT domain, and zero or more of the reducing enzymes, from which the final oxidative state of the unit is determined. ACP, acyl carrier protein; KS, β-ketoacyl synthase; AT, acyl transferase; KR, keto reductase; DH, dehydratase; ER, enoyl reductase. (B) A typical termination step of a polyketide synthase pathway. If the nucleophile that participates in the Claisen condensation is a hydroxyl group from the molecule being built, a cyclic product is produced. If the nucleophile is water, then a linear product is produced. TE, thioesterase. (C) A typical methylation step. The protons shown are labile and the resulting carbanion initiates nucleophilic attack of the activated methyl group of *S*-adenosylmethionine, leading to methylation. MT, methyl transferase.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1. *Formation of Thiazole- and Oxazole-Based Rings*

Many cyanobacterial peptides and alkaloids contain thiazole and oxazole structures within their main chains. These structures occur within both ribosomally encoded and NRPS-produced peptides. Studies have shown that these small heterocyclic rings can be formed by two distinct mechanisms.

The formation of thiazoles and oxazoles in ribosomal peptides (Fig. 3A) has been reviewed (Roy *et al.*, 1999). Most of the work has been done on the enzymes that form the bacterial product microcin B17. Heterocyclization occurs posttranslationally and appears to require zinc and the hydrolysis of approximately five units of ATP to proceed (Milne *et al.*, 1998, 1999). Binding of the polypeptide to the modifying enzymes also requires the presence of a propeptide region that is later cleaved en route to the formation of the final product (Madison *et al.*, 1997; Roy *et al.*, 1998). The ring is formed by the nucleophilic attack of the side chain thiol (Cys) or hydroxyl (Ser or Thr) group on the carbonyl of the preceding amino acid. Dehydration proceeds spontaneously to give the thiazoline (Cys) or oxazoline (Ser or Thr). The intermediate can be enzymatically oxidized to give the thiazole (Cys) or oxazole (Ser or Thr); or reduced to give the thiazolidine (Cys) or oxazolidine (Ser or Thr).

In NRPS products, the heterocyclic rings are formed as the peptide is synthesized (Fig. 3B) (Crosa and Walsh, 2002; Roy *et al.*, 1999). Most studies for NRPS heterocyclizations have involved bacterial siderophores such as mycobactin, yersiniabactin, enterobactin, and pyochelin or the myxobacteria-derived epothilones. The domains responsible for heterocycle formation are known as cyclization (Cy or C′) domains and are homologous to C domains. The Cy domains catalyze the formation of normal peptide bonds and the subsequent cyclization reaction (Chen *et al.*, 2001). As with the ribosomal products, the thiazoline or oxazoline products can be oxidized or reduced.

FIG. 2. Typical reactions catalyzed by a nonribosomal peptide synthetase. (A) A module of a nonribosomal peptide synthetase, in which each box represents a domain within a module. At each step the modules participating in the reaction are highlighted. The steps carried out are similar in concept to those carried out by a polyketide synthase, except that additional reductions or modifications of the newly incorporated unit are far less common in nonribosomal peptide synthetases. T, thiolation domain, or peptidyl carrier protein domain; C, condensation domain; A, adenylation domain. (B) A hybrid system in which a polyketide synthase module follows a nonribosomal peptide synthetase module. Note that a new carbon–carbon bond is formed between the units. (C) A hybrid system in which a nonribosomal peptide synthetase module follows a polyketide synthase module, leading to formation of a peptide bond between the two units.

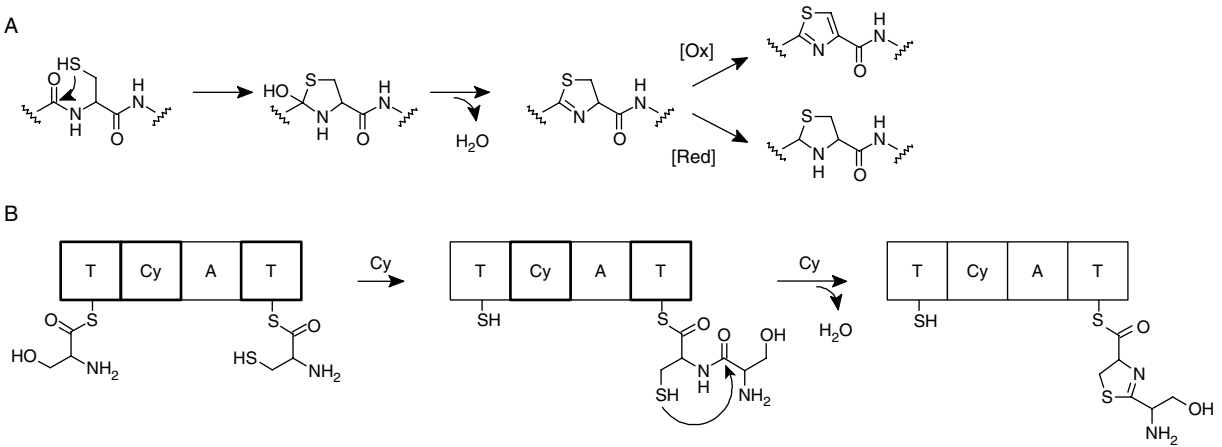

FIG. 3. Formation of thiazoles and oxazoles in peptides. (A) Thiazole formation for ribosomally encoded peptides. Heterocyclization occurs posttranslationally. (B) Thiazole formation for NRPS-produced peptides. Heterocyclization occurs as the peptide chain is assembled.

C. Connecting Product Structures to Gene Sequences

One of the most convenient features of type I linear PKSs and NRPSs is the colinearity rule: the chemical structure of the final product correlates with the sequence of modules in the biosynthetic pathway. While this rule is not universal and applies only to the sequence of subunits in the chain and does not take into account many modifications that can occur, it is still quite useful in inferring function from gene sequences and in identifying pathways responsible for a known compound.

An illustration of this is given for the microcystin biosynthetic pathway (and those of the related nodularins) (Christiansen *et al.*, 2003; Mbedi *et al.*, 2005; Moffitt and Neilan, 2004; Rouhiainen *et al.*, 2004; Tillett *et al.*, 2000). Figure 4 illustrates a hypothetical scheme for the production of microcystin. Although a phenylacetate starter unit is shown, studies indicate that the starter position can also be labeled with phenylpropanoids (Hicks *et al.*, 2006). The subsequent extension modules show good agreement in the domains present with the chemical structure of the Adda moiety. For example, when ER domains are lacking, the unit contains a double bond; when MT domains are present (as CM domains), the unit contains a pendant methyl group. Likewise, the specificity pocket sequences present in the A domains of several NRPS modules match up with the incorporated residues in the peptide portion.

However, trying to predict the structure of the product from the gene sequence alone is not as straightforward with PKSs or NRPSs of even moderate complexity. The biosynthetic genes are often distributed among multiple operons, making it difficult to predict the order of assembly of the multifunctional proteins, which is likely determined by protein–protein interactions. Moreover, many ''tailoring'' enzymes are often present which perform ''custom'' modifications of the nascent chain. In the *mcy* pathway of microcystin production, only three such tailoring enzymes are employed: *McyF*, *McyI*, and *McyJ*. But in other pathways such as the pseudomonic acid pathway in *Pseudomonas* (El-Sayed *et al.*, 2003), there can be so many tailoring enzymes as to make assignment of function difficult, even when the structure of the product is known. In addition, some of the gatekeeper enzymes may have low selectivity. As will be discussed in Section IV.D.2, there are dozens of microcystin variants that vary in amino acid substitution at two specific positions.

Nevertheless, having a gene sequence can provide useful information for identifying and isolating novel natural products from organisms whose genomic sequences have been determined. Genome mining of

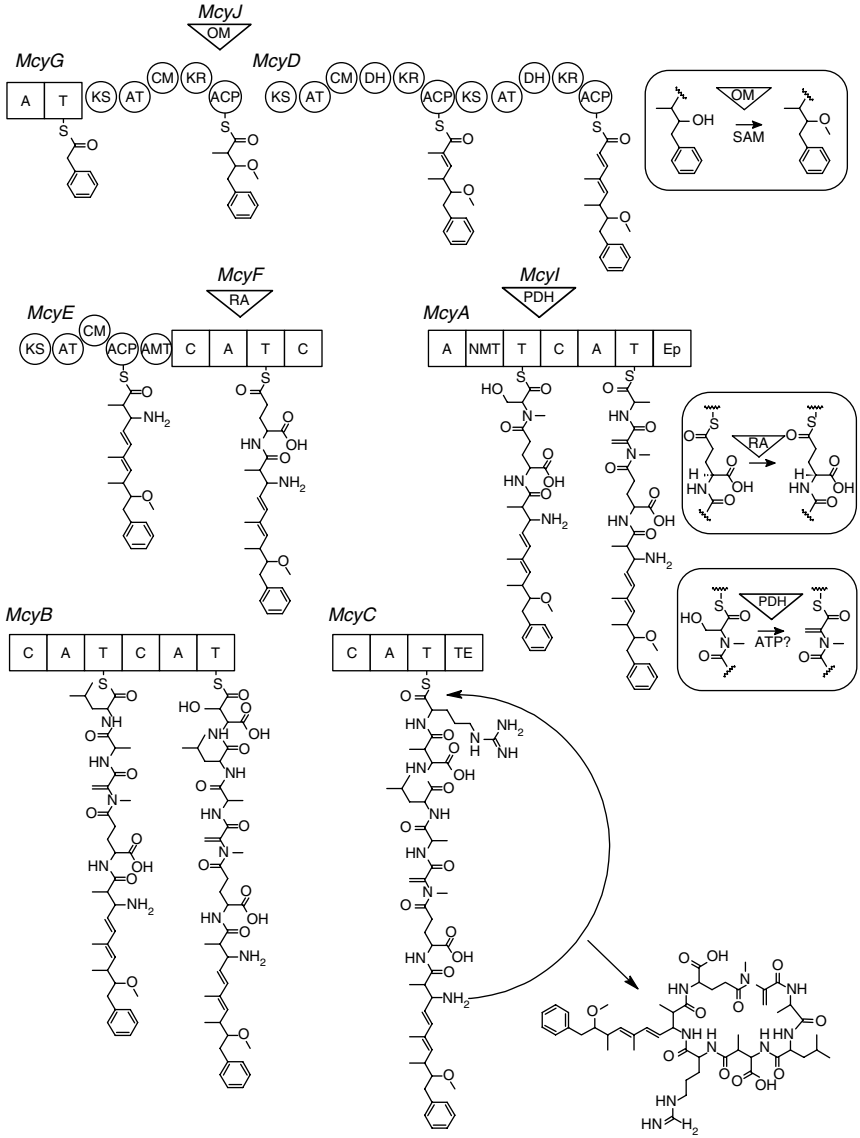

FIG. 4. The microcystin biosynthetic pathway. This figure illustrates how the domain structure of the microcystin biosynthetic enzymes correlates with the structure of the final product. Each gene is labeled in italics. The putative intermediate structure present at each module is shown. Additional enzymatic activities are shown in boxes on the right for reactions not illustrated in Figs. 1 and 2. *N*-methyl transferases are similar to *C*-methyl transferases except the target atom is nitrogen rather than carbon. Racemases,

the actinomycete *Streptomyces coelicolor* led to the identification of a putative NRPS. By using a model that maps residue substitutions within the selectivity pocket of the A domains to amino acid selectivity (Challis *et al.*, 2000), it was possible to predict the structure of the putative biosynthetic product (Challis and Ravel, 2000) which was later isolated and confirmed as a novel natural product (Lautru *et al.*, 2005). A study with the cyanobacterium *Trichodesmium* (Sudek *et al.*, 2006) led to the identification of genes showing high homology to the previously identified biosynthetic pathway for the cyclic ribosomally encoded thiazole-containing peptide patellamide from *Prochloron* (Schmidt *et al.*, 2005). On the basis of detailed analysis of the two pathways, a structure for a new natural product trichamide was predicted and provisionally confirmed by mass spectrometric analysis of extracts of *Trichodesmium* (Sudek *et al.*, 2006).

III. Polyketides

There is considerable diversity in the structures of polyketides from cyanobacteria. The structural complexity of some is comparable to that observed in the actinomycetes, from which many more polyketides have been isolated. Some of the more notable features include many instances of apparent decarboxylation of terminal units, the presence of rare *E* double bonds in fatty acids, formation of rare classes of compounds such as cyclophanes and Boeseken complexes, extensive methylation of nascent chains with SAM, and chlorination of terminal olefin bonds. Another interesting feature perhaps of evolutionary significance is the co-occurrence of similar polyketides in both cyanobacteria and actinomycetes.

A word of caution is in order for this and all subsequent sections. As is the case for most bacteria, the taxonomic classification of the cyanobacteria is not fully determined and is subject to revision. For example, several species originally belonging to *Oscillatoria* are now classified as belonging to *Planktothrix* (Suda *et al.*, 2002). The origin of metabolites will be given as originally reported without consideration to later taxonomic revisions.

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like epimerases, catalyze a reversal of configuration for a stereocenter. The final product, microcystin-LR, is shown in the lower right hand corner. The following abbreviations are in addition to those listed in Figs. 1 and 2: CM, *C*-methyl transferase; OM, *O*-methyl transferase; AMT, aminotransferase; RA, racemase; NMT, *N*-methyl transferase; PDH, 3-phosphoglyceratedehydrogenase; Ep, epimerase. [Adapted from Tillett *et al.*, 2000 with permission from Elsevier.]

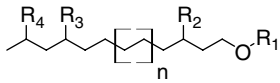
A. Linear Polyketides

1. *Hydrocarbons*

7-Methylheptadecane and 6-methylheptadecane have been reported from various genera including *Nostoc* (Calvin and Han, 1970), *Microcoleus* (Dembitsky *et al.*, 2000, 2001), *Anabaena* (Fehler and Light, 1970), and many others (Dobson *et al.*, 1988; Saiz-Jimenez *et al.*, 1990; Summons, 1987; Tsuchiya and Matsumoto, 1988; Tsuchiya *et al.*, 1981; Zeng *et al.*, 1992). These compounds have been reported to act as pheromones in various insects. Such compounds are most likely derived from fatty acid pathways via decarboxylation of the acid head group (McInnes *et al.*, 1980).

2. *Long-Chain Alcohols*

The heterocyst glycolipids (Fig. 5) are a class of lipids found in the envelope of cyanobacterial heterocysts, a differentiated type capable of fixing nitrogen. Studies on heterocyst glycolipid composition have been

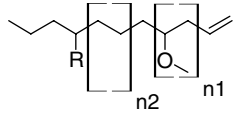


Genus	R_1	R_2	R_3	R_4	n
Anabaena	Glc	OH	H	OH	8
	Gal	OH	H	OH	9
	Glc	=O	H	OH	9
	Gal	=O	OH	OH	10
Calothrix	Glc	OH	=O	OH	9
	Man	OH	=O	OH	9
Chlorogloeopsis	Man	OH	OH	OH	11
	Glc	OH	=O	OH	11
Cyanospira	Glc	OH	H	OH	9
	Glc	OH	H	=O	9
Fischerella	Glc	OH	OH	OH	11
	Glc	OH	=O	OH	11
Microchaete	Glc	OH	H	OH	9
	Glc	=O	H	OH	9
Nodularia	Glc	OH	H	OH	8
	Glc	=O	H	OH	8
Tolypothrix	Glc	OH	H	OH	9
	GlcB	OH	H	OH	9
	Glc	=O	OH	OH	10

Fig. 5. Heterocyst glycolipids from cyanobacteria. Not all known examples are shown. However, enough examples are shown for each genus to illustrate the diversity in size and oxygenation patterns observed. Glc, α-D-glucopyranosyl; GlcB, β-D-glucopyranosyl; Gal, α-D-galactopyranosyl; Man, α-D-mannopyranosyl.

reported for *Anabaena* (Gambacorta *et al.*, 1996; Soriente *et al.*, 1995), *Calothrix* (Gambacorta *et al.*, 1998), *Chlorogloeopsis* (Gambacorta *et al.*, 1998), *Cyanospira* (Soriente *et al.*, 1993), *Fischerella* (Gambacorta *et al.*, 1998), *Microchaete* (Gambacorta *et al.*, 1998), *Nodularia* (Soriente *et al.*, 1992), *Scytonema* (Gambacorta *et al.*, 1998), and *Tolypothrix* (Gambacorta *et al.*, 1998). Structurally, the heterocyst glycolipids consist of a multiply hydroxylated long-chain glycoside. The chain length can be even values between 26 and 32 carbons. Glycosylation occurs at the head of the chain, and glucose, galactose, and mannose groups have all been reported. Oxygenation occurs at positions 1 and 3 as well as at ω2 and often at ω4 in the form of carbonyls or alcohols. Transposon mutagenesis studies with *Nostoc punctiforme* led to the identification of a putative multifunctional PKS-like gene apparently responsible for synthesis of the heterocyst glycolipids (Campbell *et al.*, 1997), a result consistent with earlier acetate-labeling studies (Gambacorta *et al.*, 1995).

A series of isotactic polymethoxy-1-alkenes (Fig. 6) have been reported from *Tolypothrix* (Mori *et al.*, 1991b), *Scytonema* (Mori *et al.*, 1991a), and *Aphanizomenon* (Banker *et al.*, 2000). Determination of absolute stereochemistry by synthesis indicated stereoselective reduction from the same face of all oxygenated atoms (Mori *et al.*, 1991a,b). The chain length of these hydrocarbons varies among the odd number values between 17 and 31, suggesting that they arise by decarboxylation of the appropriate long-chain fatty acid precursor, a process previously identified to occur in the cyanobacterium *Anacystis nidulans* (McInnes *et al.*, 1980).



Genus	n1	n2	R
Aphanizomenon	12	0	H
	11	0	H
Scytonema	5	0	H
	6	0	H
	6	2	OCH_3
	7	2	OCH_3
	8	0	H
	8	2	OCH_3
	9	0	H
Tolypothrix	10	0	H

FIG. 6. Isotactic polymethoxy-1-alkenes from cyanobacteria.

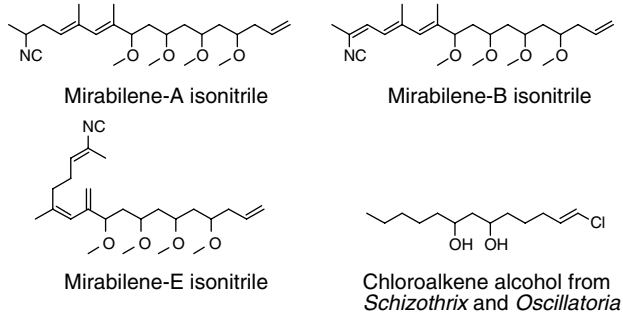


FIG. 7. The mirabilene isonitriles and a chloroalkene discussed in the text.

The mirabilene isonitriles (Fig. 7) from *Scytonema* (Carmeli *et al.*, 1990a) are a class of mildly cytotoxic compounds very similar to the isotactic polymethoxy-1-alkenes described above, but contain pendant methyl groups, additional olefinic bonds, and an isonitrile substituent at ω2. In addition to the ones shown, other congeners representing several double bond configurational isomers of mirabilene-B isonitrile also exist. The positioning of the methyl groups relative to the oxygenation pattern suggests that they result from incorporation of propionate or from SAM-mediated methylation. There are many potential sources of the isonitrile group in these molecules. Direct incorporation of cyanide is possible as found in sponge terpenoids (Garson and Simpson, 2004) given that cyanobacteria can produce cyanide from histidine in dark reactions (Pistorius *et al.*, 1979).

A short-chain chloroalkene alcohol (Fig. 7) has been reported from a mixture composed primarily of *Schizothrix* and *Oscillatoria* (Mynderse and Moore, 1978b). This compound also has an odd number of carbons and an alkene head group.

3. *Fatty Acids*

In addition to the usual suite of primary fatty acids, more elaborate variants have been reported from various species. For example, *Lyngbya* cyanobacteria have been reported to produce unusual branched chain fatty acids. The acyl chain of the grenadadienes (Sitachitta and Gerwick, 1998) contains a cyclopropyl ring probably derived from SAM-mediated methylation of an olefinic bond (Fontecave *et al.*, 2004), as shown in Fig. 8A. The biogenetic origin of the pentadienyl diol moiety is not apparent, and in grenadadiene itself, this moiety also contains a vinylic bromine group. A related molecule with a brominated propenyl diol

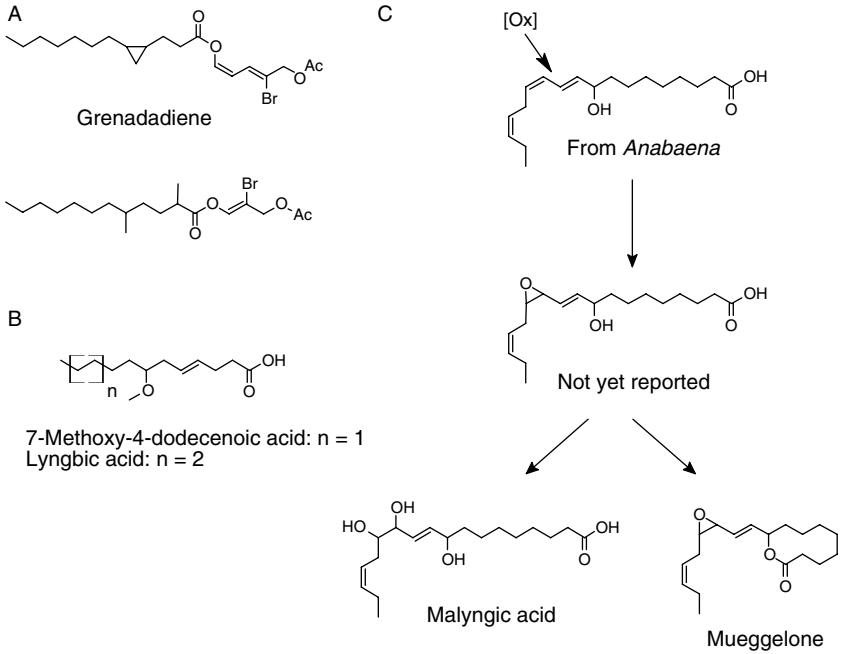


FIG. 8. Some of the more unusual fatty acids reported from cyanobacteria. (A) Grenadadiene and a similar fatty acid from *Lyngbya*. (B) Two unsaturated fatty acid ethers from *Lyngbya*. (C) A putative biosynthetic pathway relating a polyunsaturated fatty acid found in *Anabaena* to the *Lyngbya* products malyngic acid and mueggelone.

moiety and without the cyclopropyl group was also reported from *Lyngbya* (Hodder and Capon, 1991a).

A series of C_{12} through C_{14} unsaturated, hydroxylated fatty acid derivatives have been reported from *Lyngbya* (Cardellina *et al.*, 1978; Mesguiche *et al.*, 1999). The largest family contains a double bond at position 4 and an oxygen at position 7 (Fig. 8B). In spite of their simplicity, these compounds have been reported to have immunosuppressive activity (Virolleaud *et al.*, 2006). They are also of interest as potential precursors for hermitamides and malyngamides (Section V.B.1). Malyngic acid is another hydroxylated, unsaturated acid from *Lyngbya* (Cardellina and Moore, 1980) that contains a distinctive 1,2-diol moiety. It is interesting to note that, on paper and ignoring stereochemistry, malyngic acid can be derived from another fatty acid reported from *Anabaena* (Murakami *et al.*, 1992) via epoxidation of a conjugated

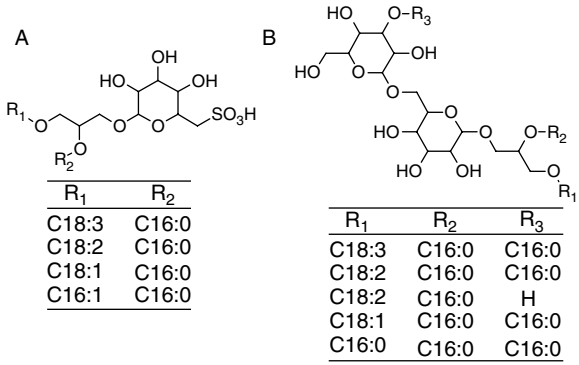
double bond followed by hydrolytic ring opening (Fig. 8C). The lactone mueggelone isolated from *Aphanizomenon* (Papendorf *et al.*, 1997) and *Gloeotrichia* (Stierle *et al.*, 1998) appears to be related to malyngic acid.

4. *Glycolipids*

A family of sulfolipids with anti-HIV activity was reported from cyanobacteria (Fig. 9A) (Gordon and Danishefsky, 1992; Gustafson *et al.*, 1989). Other examples (not shown) have been isolated from *Scytonema* as well (Reshef *et al.*, 1997). The compounds are diacylglycerols with a sulfoquinovosyl head group. These types of lipids make excellent detergents and are found (though neither exclusively nor universally, but exceptions to either case are rare) in the photosynthetic membranes of plants, algae, and other photosynthetic organisms (Benning, 1998). Synthesis of the head group is believed to proceed through UDP-glucose, and putative genes required for the production of sulfolipids in the cyanobacterium *Synechococcus* have been cloned (Güler *et al.*, 1996, 2000).

Galactolipids are another class of lipids required in the thylakoid membrane of various photosynthetic organisms. Various monoacyl and diacyl galactolipids (not shown) have been identified in *Phormidium* (Murakami *et al.*, 1991b), *Oscillatoria* (Son *et al.*, 2000, 2001), among others, and biosynthesis of these galactolipids in cyanobacteria proceeds via epimerization of glucosyl diacylglycerol. A gene catalyzing formation of this precursor has been identified and cloned from *Synechocystis* and *Anabaena* (Awai *et al.*, 2006).

An interesting series of diglycolipids were isolated from *Oscillatoria* (Fig. 9B) (Reshef *et al.*, 1997), and members of this family have fatty acyl



R_1	R_2
C18:3	C16:0
C18:2	C16:0
C18:1	C16:0
C16:1	C16:0

R_1	R_2	R_3
C18:3	C16:0	C16:0
C18:2	C16:0	C16:0
C18:2	C16:0	H
C18:1	C16:0	C16:0
C16:0	C16:0	C16:0

FIG. 9. Some of the glycolipid metabolites from cyanobacteria. (A) Sulfoglycolipids. (B) Diglycolipids from *Oscillatoria*.

groups attached to a sugar alcohol group in addition to the glycerol alcohols. Other diglycolipids have been isolated from other cyanobacteria (structures not shown) (Bruno *et al.*, 2005; Murakami *et al.*, 1991b).

B. Cyclic Polyketides

1. *Small Ring Systems*

An assemblage of coral and a *Synechocystis* cyanobacterium was reported to contain the cytotoxic nakienones and the related metabolite nakitriol (Fig. 10A) (Nagle and Gerwick, 1995). These compounds contain small five- or six-membered rings with a branched side chain. Indeed, comparing the structures suggests a common linear polyketide precursor that can condense to form either a five- or six-membered ring. A plausible biogenetic pathway might involve an α,β-diketo intermediate (Fig. 10A).

Kalkipyrone was reported from an assemblage of *Lyngbya* and *Tolypothrix* (Graber and Gerwick, 1998) and consists of a methylated γ-pyrone head with a methylated side chain (Fig. 10B). Although the side chain resembles an isoprenoid, the structure might also be derived from a methylated polyketide with a pyruvate starter unit.

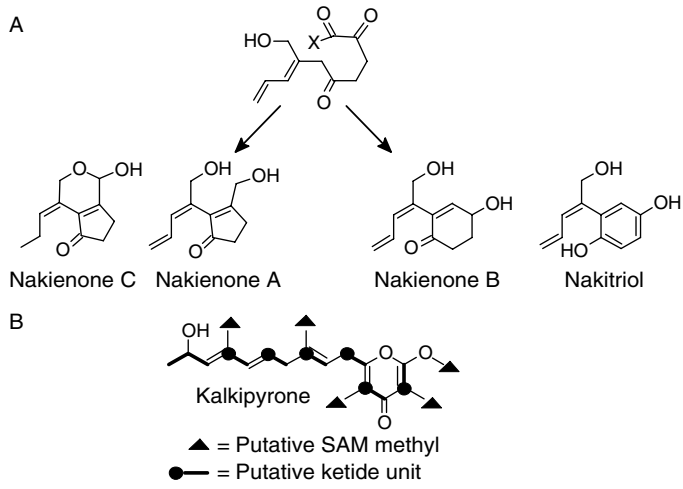


Fig. 10. Various ring-containing polyketides from cyanobacteria. (A) A possible biogenesis for the nakienones from a putative α,β-diketo polyketide intermediate. Only a few of all reported nakienones are shown. (B) Suggested biogenesis of kalkipyrone. Putative acetate units are labeled as thick bonds with methyl groups marked with circles. The starter unit could be pyruvate or 2-hydroxypropionate. An alternate biosynthesis using a geranyl-derived unit is also possible.

2. *Aromatic Polyketides*

The hierridins (Fig. 11A) are a simple class of long-chain alkylated phenols found in *Phormidium* (Papendorf *et al.*, 1998). The long alkyl chain is not branched. The hierridins contain only a single aromatic ring per polyketide chain in what appears to be a common trait for such compounds in cyanobacteria. The hierridins all contain an odd number of carbons, suggesting decarboxylation of the polyketide chain or possible incorporation of propionate. They resemble a metabolite reported from the lichen *Ramalina hierrensis* (Gonzalez *et al.*, 1992).

Cyclophanes are a class of macrocyclic compounds in which a hydrocarbon chain connects nonadjacent sites on an aromatic ring. The few biologically produced cyclophanes are found exclusively in cyanobacteria. The first reported were the cylindrocyclophanes (Fig. 11B) from *Cylindrospermum* and the chlorinated nostocyclophanes from *Nostoc* (Chen *et al.*, 1991; Moore *et al.*, 1990, 1992a). A monomeric cyclophane containing an alkyne bond, nostocyclyne A (Fig. 11B), was reported from *Nostoc* (Ploutno and Carmeli, 2000). Note the similarity in structure of nostocyclyne A to the linear hierridin A.

The biosynthesis of the cylindrocyclophanes has been examined. Biosynthetic feeding studies using isotopically labeled acetate revealed that cylindrocyclophanes are produced as nonaketides that undergo

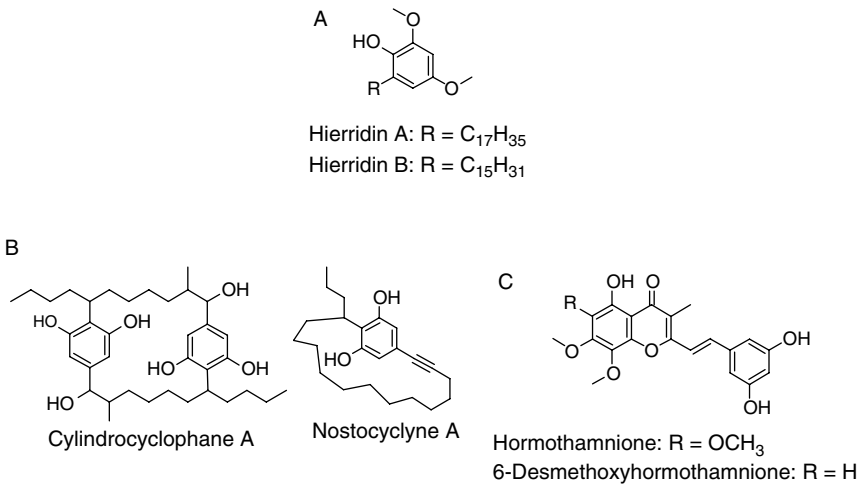


FIG. 11. Various aromatic polyketides from cyanobacteria. (A) Hierridins A and B. (B) Cyclophanes from cyanobacteria. Related derivatives of the structures shown are also known. (C) Hormothamniones.

internal cyclization to form the aromatic rings and dimerization to form the final compounds (Bobzin and Moore, 1993). The methyl groups are derived from C2 of acetate, a phenomenon observed in some dinoflagellate polyketides (Macpherson *et al.*, 2003; Sato *et al.*, 2000). Incorporation of pendant methyl groups derived from C2 of acetate typically occurs at electrophilic carbons in the nascent polyketide chain, usually derived from C1 of acetate. A similar phenomenon has been observed in the dinoflagellates and the bacterium *Pseudomonas* where a modified hydroxymethylglutarate (HMG) pathway has been suggested (El-Sayed *et al.*, 2003). Methylation at C1-derived carbons of the polyketide chain occurs often in cyanobacterial polyketide pathways as indicated throughout this chapter.

The highly oxygenated chromone-containing hormothamniones (Fig. 11C) were isolated from *Hormothamnion* (Gerwick, 1989; Gerwick *et al.*, 1986). They were reported as being potent cytotoxins against cancer cells. Acetyl esters of hormothaminone exhibit antioxidant (Takamatsu *et al.*, 2003) and anticell-adhesion activities (Takamatsu *et al.*, 2004). These compounds may be derived from pathways similar to the type III PKS chalcone synthases in plants and bacteria (Austin and Noel, 2003; Funa *et al.*, 1999; Moore and Hopke, 2001). The type III PKS systems, which act iteratively, are chemically distinct from other PKSs in that they use acyl-coenzyme A as extender units.

The oscillatoxins are severe skin irritants found in *Oscillatoria* and *Schizothrix* (Mynderse and Moore, 1978c) and in *Lyngbya* (Entzeroth *et al.*, 1985; Moore *et al.*, 1984a). The oscillatoxins occur in two different forms: one contains a cyclohexane/tetrahydropyran spiro ring system, the other a tetrahydropyran/tetrahydropyran spiro ring system that is part of a macrocyclic lactone (Fig. 12). The latter form is also found in the closely related aplysiatoxins, originally reported from the sea hare *Stylocheilus* (Kato and Scheuer, 1974) which is known to graze on *Lyngbya*. Both forms are derived from the same polyketide precursor via alternative cyclizations to form the different spiro ring systems. Both forms also include analogues that exhibit bromination or variable presence of a terminal methyl group on the C_4/C_5 acyl chain.

3. *Lactones*

Malyngolide (Cardellina *et al.*, 1979d) and tanikolide (Singh *et al.*, 1999) are two small lactones that have been reported from *Lyngbya* (Fig. 13A). While their biogenesis appears to follow the usual polyketide process, the pendant hydroxymethyl group is introduced at an electrophilic carbon of the putative polyketide chain and could follow a pathway similar to that found in the cyclophanes (Section III.B.2).

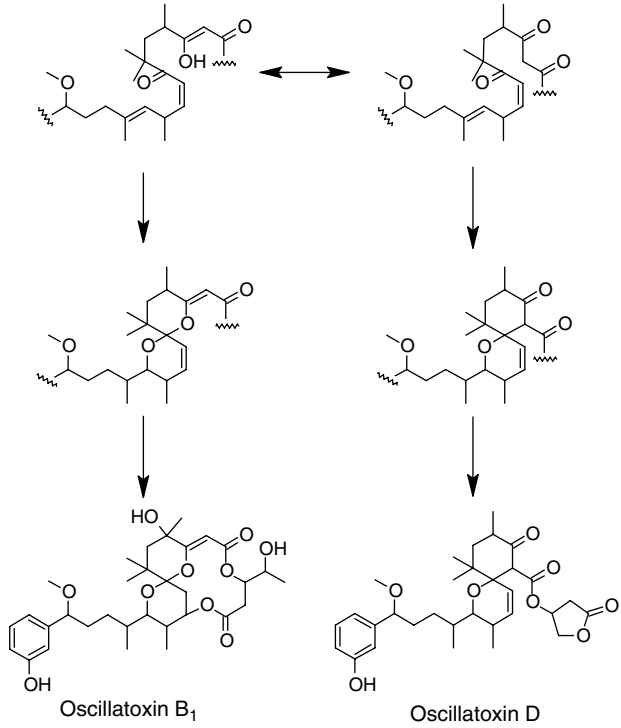


FIG. 12. Alternative condensation pathways leading to the two main structural families of the oscillatoxins.

Interestingly, the two compounds are of opposite configurations at the quaternary carbon.

A series of complex macrolides and their glycosides (Fig. 13B) have been isolated from cyanobacteria. The macrolide acutiphycin from *Oscillatoria* is a cytotoxic compound that contains a pyran ring (Barchi *et al.*, 1984) and is somewhat reminiscent of the amphidinolides found in a dinoflagellate (Kobayashi and Tsuda, 2004). The glycoside derivatives were isolated from *Lyngbya* and contain methylated rhamnose moieties. These compounds bear strong similarity to the callipeltosides from a lithistid sponge (Zampella *et al.*, 1996, 1997), the aurisides from a mollusk (Sone *et al.*, 1996), and the polycavernosides from a red alga (Yotsu-Yamashita *et al.*, 1993, 1995). Lyngbyaloside (Klein *et al.*, 1997) has a 16-membered ring and includes a terminal bromoalkene moiety. A related compound, lyngbyaloside B (Luesch *et al.*, 2002c), contains a 14-membered ring, a slightly different pattern of chain methylation,

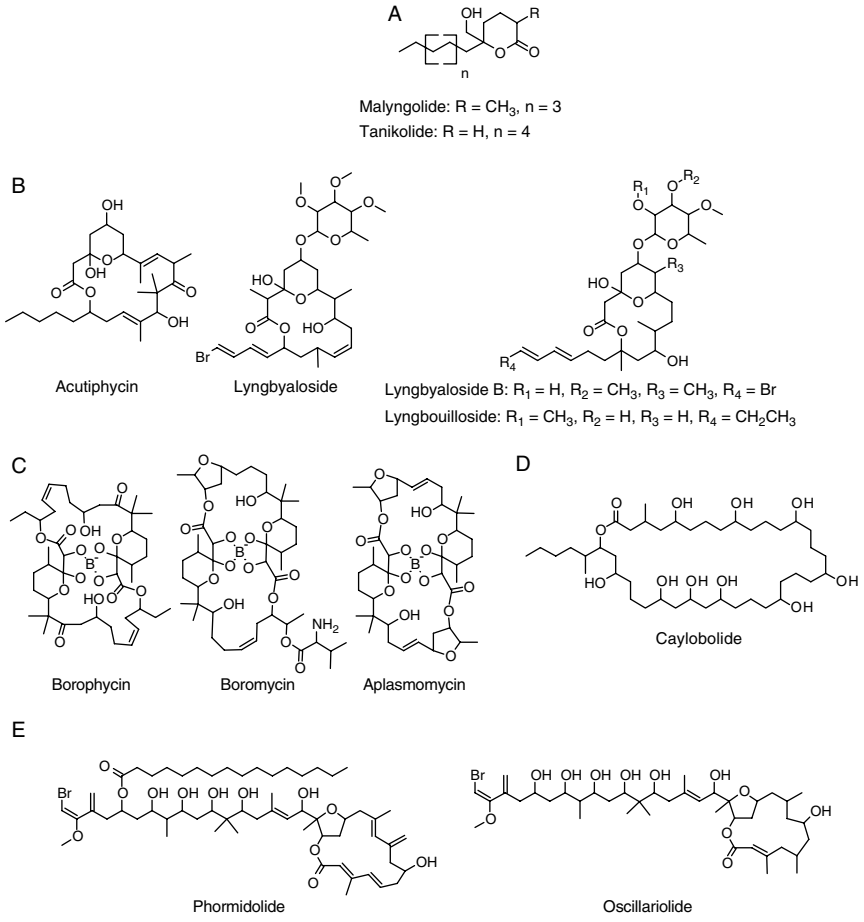


FIG. 13. Several macrolides reported from cyanobacteria. The nitrogen-containing scytophycins are discussed in the alkaloid section. (A) Malyngolide and tanikolide. (B) Tetrahydropyran-containing macrolides. (C) The cyanobacterial metabolite, borophycin. Also shown are similar compounds from *Streptomyces*, boromycin and aplasmomycin. (D) Caylobolide, a polyhydroxy macrolide. (E) The tetrahydrofuran-containing macrolides phormidolide and oscillariolide.

and a terminal brominated conjugated diene. Lyngbouilloside (Tan *et al.*, 2002) is nearly identical to lyngbyaloside B, differing only in replacement of the bromine moiety with an ethyl group, and in the methylation pattern of the sugar. The macrocyclic ring is most plausibly polyketide derived, and whereas acutiphycin is likely assembled from

11 intact acetate units, all of the lyngbyaloside-like compounds contain an odd number of chain carbons. Since these compounds appear not to have undergone decarboxylation, it suggests the incorporation of a propionate starter unit or deletion of a carbon from the nascent polyketide chain as occurs in various dinoflagellate polyketides (Kobayashi *et al.*, 1995, 2001; Kubota *et al.*, 2001; Sato *et al.*, 2000; Tsuda *et al.*, 2001, 2002; Wright *et al.*, 1996).

Borophycin from *Nostoc* (Fig. 13C) is a polyketide Boeseken complex of boric acid, a rare structural class among naturally occurring compounds (Banker and Carmeli, 1998; Hemscheidt *et al.*, 1994). Similar compounds include boromycin (Dunitz *et al.*, 1971; Huetter *et al.*, 1967; Lee *et al.*, 1985) and aplasmomycin (Nakamura *et al.*, 1977; Okami *et al.*, 1976; Sato *et al.*, 1978), both from *Streptomyces*, and tartrolon B (Schummer *et al.*, 1996) from the gliding bacterium *Sorangium*. The structural similarities between the cyanobacterial product borophycin and the streptomycete products boromycin and aplasmomycin are striking. Borophycin and aplasmomycin are both diolides, or dimeric macrolides. Boromycin is asymmetric in that one of its polyketide chains lacks a furan ring but is instead esterified by valine. Labeling studies showed that in the case of boromycin and aplasmomycin, most of the main chain carbon atoms are derived from acetate and the pendant methyl groups derived from methionine (Chen *et al.*, 1979, 1980, 1981a,b; Lee *et al.*, 1987). The starter unit, however, was found to be derived from the metabolic C_3 pool, probably phosphoenol pyruvate or phosphoglyceric acid based on extensive studies with feeding various potential precursors as well as studies with stereospecifically deuterium- or tritium-labeled glycerol substrates. Similar results were seen for borophycin, except that the starter unit appears to be derived from acetate to which a methionine-derived methyl group is added. The structural similarities and sporadic distribution of these compounds suggest that the biosynthetic genes may have been involved in lateral gene transfer (LGT). In fact, an example of LGT was reported between cyanobacteria and dinoflagellates (Waller *et al.*, 2006).

Caylobolide (Fig. 13D) is a cytotoxic macrolide containing a 36-membered ring from *Lyngbya* (MacMillan and Molinski, 2002). It is plausibly derived from a single polyketide chain assembled from 20 acetate units. There are two pendant methyl groups, one of which is attached to a carbon likely derived from the carbonyl of acetate.

Oscillariolide (Murakami *et al.*, 1991a) and phormidolide (Williamson *et al.*, 2002), isolated from *Oscillatoria* and *Phormidium*, respectively, are another family of macrolides containing a terminal bromoalkene moiety (Fig. 13E). Both compounds contain furan rings within the macrocycle.

They contain nearly identical side chain groups with the exception of an extra palmitoyl substituent on phormidolide. Like the lyngbyalosides, these macrolides contain an odd number of chain carbons, suggesting similarities in the biosynthetic pathways producing these two sets of compounds. Distinctive features of oscillariolide and phormidolide include the polyhydroxy side chain and the presence of pendant methyl groups at chain carbons derived from both C1 and C2 of acetate.

IV. Cyanopeptides

A. Introduction

Both marine and freshwater cyanobacteria have the ability to produce a large number of peptide metabolites. These cyanopeptide metabolites have enormous structural diversity, ranging from linear to cyclic and multicyclic with simple peptide, depsipeptide, and lipopeptide structures encompassing a size range of 300–2000 Da. Many peptides contain nonproteinogenic residues, like D-amino acids, β-amino acids, hydroxy acids, and *N*-methylated acids, in addition to proteinogenic amino acids, which add further to their structural diversity. As described in Section II.B, the majority of these cyanobacterial peptides are not ribosomally synthesized but instead are synthesized by NRPSs (Von Dohren *et al.*, 1999). Most cyanobacterial peptide biosynthetic pathways have yet to be studied, but the work that has been done reveals several cyanopeptides (microcystins, anabaenapeptolides, nostopeptolides, and nostocyclopeptides) result from hybrid biosynthetic schemes, incorporating NRPSs and PKSs, as well as numerous tailoring enzymes (Fig. 4) (Dittmann *et al.*, 2001b). These multifunctional enzymes assemble the backbone of the metabolite and additional tailoring enzymes involved in epimerization, *N*- and *O*-methylation, oxidation, reduction, dehydration, sulfation, halogenation, and heterocyclization (Tillett *et al.*, 2000; Welker and von Doehren, 2006) result in the characteristic motifs of each peptide family.

The most common producers of cyanopeptide metabolites belong to members of the Oscillatoriales and Nostacales, followed by Chroococcales and Stigonematales. However, it has been suggested that this species distribution reflects availability of biomass and culturability of cyanobacterial strains rather than a true indication of metabolite production across all cyanobacterial genera (Welker and von Doehren, 2006). Additionally, numerous peptide metabolites that have previously been isolated from marine macroorganisms are speculated to be produced by microbial or cyanobacterial symbionts (e.g., dolastatins

originally isolated from the sea hare *Dolabella* have recently been isolated from the marine cyanobacteria *Symploca* sp.). Only those peptide metabolites that have been isolated from cyanobacteria directly will be considered in this chapter.

B. Classification and Nomenclature

Classification of cyanopeptide metabolites has proven to be very difficult due to their immense structural diversity and species distribution. Cyanopeptides are commonly named using a prefix corresponding to the origin of the metabolite (species or location) and a suffix corresponding to the structural class. However, there are several major problems with the current classification scheme for cyanopeptides that can confuse the casual reader: (1) The simultaneous publication of many cyanopeptide metabolites with the same structural features from different cyanobacterial genera, resulting in overlaps in classifications such as microginins, oscillaginins, and nostoginins, all having the same basic structure but isolated from *Microcystis*, *Oscillatoria*, and *Nostoc* respectively. (2) Several names in the literature for one structural class. For instance the cyanobacterial depsipeptides containing a unique 3-amino-6-hydroxy-2-piperidone (Ahp) residue are referred to as cyanopeptolins (nodulapeptolins, oscillapeptolins), micropeptins (aeruginopeptins, nostapeptins, planktopeptin, oscillapeptin, tasipeptin), anabaenapeptolides (oscillapeptolides, nostapeptolides), nostocyclin, microcystilide, scyptolin, somamides, and hofmannolin. (3) Some structural classes, such as the dolastatins, encompass several classes of structurally unrelated compounds, including both linear and cyclic peptides with variable numbers of amino acid residues. (4) Cyanobacterial peptides with names already used to describe completely unrelated compounds in other organisms. For example, the name microcin is used to describe a linear protease inhibitory peptide from *Microcystis* and also an antimicrobial ribosomal peptide produced by *Escherichia coli* (Banker and Carmeli, 1999; Salomon and Farias, 1992). (5) Discrepancies in naming the acyl moieties of lipopeptides. For instance, some variants of the linear lipopeptide microginins isolated from *Nostoc* contain 3-amino-2-hydroxy-octanoic acid, a modified β-amino acid referred to as Ahoa (Fig. 16) (Ploutno and Carmeli, 2002). However, the cyclic lipopeptide nostophycin isolated from *Nostoc* contains a different modified β-amino acid, namely 3-amino-2,5-dihydroxy-8-phenyl-octanoic acid, which is also referred to as Ahoa (Fig. 22) (Fujii *et al.*, 1999). While such nomenclature problems cause considerable confusion, it is not the intention of this chapter to address these problems, but instead to acknowledge

that they exist and caution the reader accordingly. The metabolite names as well as the names of their amino acids and other constituents described in this chapter will not be altered from those names found in the original texts.

For the purposes of this chapter, cyanopeptide metabolites will first be divided into linear and cyclic peptides. Cyclic cyanopeptides will be further classified as peptides or depsipeptides. In each cyanopeptide subdivision, peptides will be discussed in order of increasing size when possible. Flat structures are depicted, with no consideration of stereochemistry. For more detailed information, the reader is directed to the original publications.

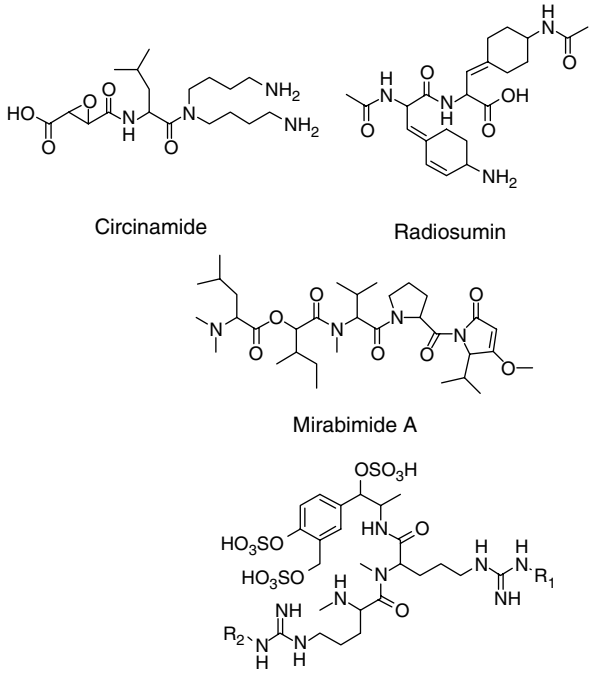
C. Linear Peptides

1. *Simple Linear Peptides*

Circinamide, isolated from *Anabaena circinalis*, is composed of 2,3-epoxy succinic acid, Leu, and homospermidine (Fig. 14). Homospermidine is a rare triamine that has been found to be the major polyamine of nitrogen fixing cyanobacteria (Hamana *et al.*, 1983). Circinamide is the first papain inhibitor reported from cyanobacteria and is related to E-64, a potent protease inhibitor isolated from *Aspergillus japonicus*. The synthetic drug loxistatin was designed from a prototype of E-64 and is a clinically usable drug for the treatment of muscular dystrophy (Shin *et al.*, 1997a).

Radiosumins are trypsin inhibitory dipeptides composed of two nonproteinogenic amino acids and two *N*-acyl groups. Radiosumin (Fig. 14), originally isolated from *Plectonema radiosum*, contains the two novel amino acid constituents, 2-amino-3-(4-amino-2-cyclohexenylidene)-propionic acid (Aayp) and 2-amino-3-(4-amino-2-cyclohexylidene)propionic acid (Aacp) (Matsuda *et al.*, 1996b). Radiosumin B was later reported from *Microcystis aeruginosa* and contains Aayp and 2-amino-3-(4-aminomethyl-2-cyclohexen-1-ylidene)propionic acid (Amyp), the *N*-methyl derivative of Aayp (Coleman and Wright, 2001).

Mirabimides are cytotoxic *N*-acylpyrrolinones isolated from *Scytonema mirabile*. Mirabimide A (Fig. 14) has the structure *N*-[2-(*N,N*-dimethylleucyl)oxy-3-methylpentanoyl-*N*-methylvalyl-prolyl]-4-methoxy-5-isopropyl-3-pyrrolin-2-one and mirabimides B-D are the valyl, 2-oxy-3-methylbutanoyl, and *N*-acetyl-*N*-methylleucyl analogues, respectively (Carmeli *et al.*, 1991b). An interesting feature of the mirabimides that is common among many cyanobacterial metabolites is the occurrence of α-hydroxy analogues of proteinogenic amino acids, for example the second residue in mirabimide A, which resembles isoleucine.



Aeruginoguanidine 98-A	R_1 = prenyl	R_2 = geranyl
Aeruginoguanidine 98-B	R_1 = H	R_2 = geranyl
Aeruginoguanidine 98-C	R_1 = prenyl	R_2 = 6-hydroxy-3,7-dimethyl-2,7-octadienoic acid

FIG. 14. Structural representatives of simple linear peptides from cyanobacteria.

Such analogues are likely derived from the amino acids that they resemble. There is precedent for this in plants where 4-hydroxyphenyllactic acid is synthesized from tyrosine via reduction of the α-keto analogue of tyrosine (Kim *et al.*, 2004).

Aeruginoguanidines (Fig. 14) are cytotoxic peptides from *Microcystis aeruginosa* containing the unique constituent Hphpa trisulfate (1-(4-hydroxy-3-hydroxymethyl)-phenyl-1-hydroxy-2-propylamine 4′,3′, 1-tri-*O*-sulfate) as well as two modified arginine groups. Aeruginoguanidine 98A contains *N*-methyl-*N*-prenylarginine (MpArg) and *N*-methyl-*N*-geranylarginine (MgArg). Aeruginoguanidine 98B contains *N*-methylArg and MgArg and aeruginoguanidine 98C contains MpArg and *N*-methyl-*N*-2,6-dimethyloctylarginine (MdoArg) as well as Hphpa trisulfate (Ishida *et al.*, 2002).

Aeruginosins, as well as their derivatives microcin, oscillarin, and spumigins, are linear metabolites containing a hydroxyphenyllactic acid (Hpla) at the N-terminus, a variable proteinogenic amino acid residue, an unusual α-amino acid, 2-carboxy-6-hydroxy-octahydroindole (Choi), and a basic arginine-derived side chain at the C-terminus (Fig. 15). Numerous

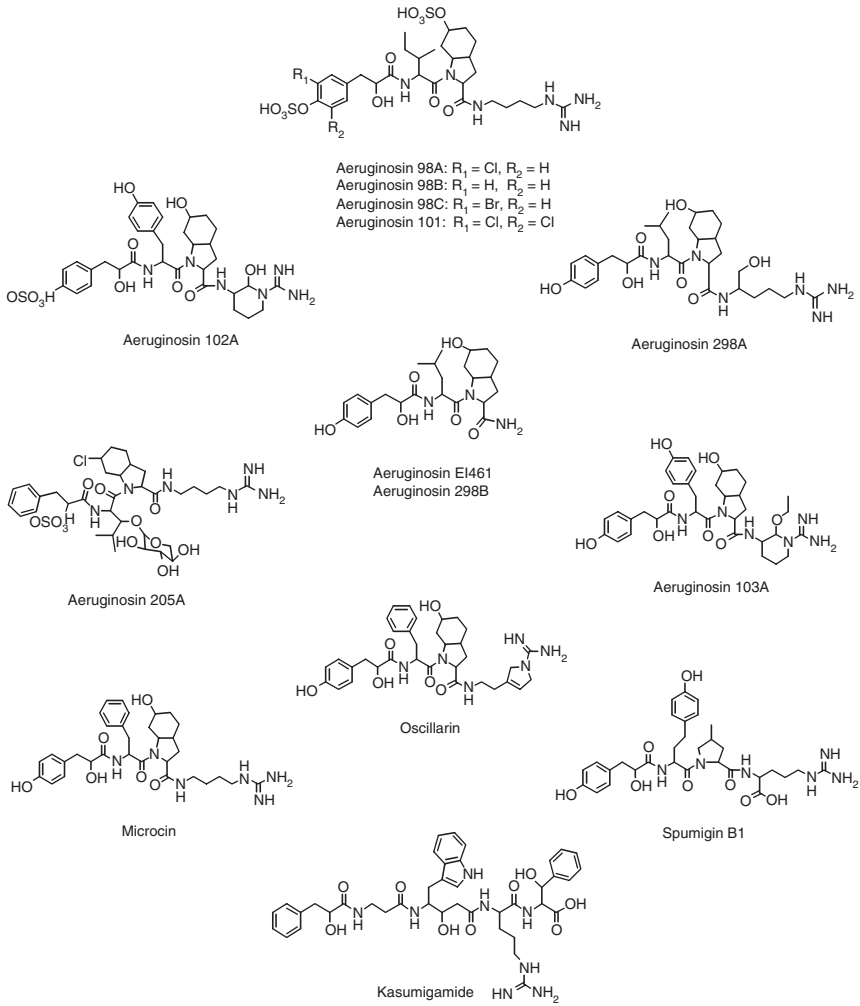


FIG. 15. Representatives of the aeruginosin family of linear peptides. Unique features of these linear cyanopeptides include an N-terminal Hpla, the unusual α-amino acid 2-carboxy-6-hydroxy-octahydroindole (Choi), and a C-terminal arginine-derived side chain.

modifications, including halogenation and sulfation, at both the Hpla and the Choi moiety, in addition to the variable C-terminus side chain account for the structural diversity of this class of cyanopeptide. Many of these variants inhibit serine proteases to varying degrees and are produced by *Microcystis* unless noted otherwise. Aeruginosins 98A-C contain sulfated Hpla with variable halogenation, Leu, a sulfated Choi residue, and the decarboxylated form of arginine, agmatine, for the N-terminus side chain. Aeruginosin 98A contains a chlorinated Hpla-sulfate, whereas aeruginosin 98B contains the nonhalogenated Hpla- sulfate and aeruginosin 98C contains a rare brominated Hpla-sulfate. Aeruginosin 101 differs in structure from aeruginosin 98A by an additional chlorine group at the N-terminal Hpla-sulfate (Ishida *et al.*, 1999; Murakami *et al.*, 1995). Aeruginosins 205A and -B are glycosylated aeruginosin derivatives isolated from *Oscillatoria*. These variants contain sulfated phenyllactic acid, a hydroxy leucine residue with an attached xylopyranose sugar, a chlorinated Choi moiety, and agmatine (Shin *et al.*, 1997b). Microcin, a trypsin inhibitor from *Microcystis*, contains Hpla, Phe, Choi, and agmatine (Banker and Carmeli, 1999). Aeruginosins 102A and -B as well as aeruginosins 89A and -B contain argininal as the basic arginine-derived side chain as well as unmodified Choi residues. Aeruginosins 102A and -B contain Hpla-sulfate and Tyr, whereas aeruginosins 89A and -B contain chlorinated Hpla-sulfate and Leu (Ishida *et al.*, 1999; Matsuda *et al.*, 1996a). Aeruginosin 298A contains Hpla, Leu, Choi, and the arginine derivative argininol. Aeruginosin 298B contains the same structural features as aeruginosin 298A but lacks the arginine-derived side chain and has a terminal primary amide (Ishida *et al.*, 1999). Aeruginosin EI461 has the same structure as aeruginosin 298B but with unspecified differences in stereochemistry (Ploutno *et al.*, 2002). Aeruginosin 103A contains Hpla, Tyr, Choi, and a unique N-terminus side chain, 1-amidino-2-ethoxy-3-aminopiperidine (Aeap) (Kodani *et al.*, 1998). Oscillarin, isolated from *Oscillatoria*, also contains the sequence Hpla-Tyr-Choi-Aeap; however, Aeap in oscillarin represents 1-amidino-3-(2-aminoethyl)-3-pyrroline, an arginine derivative also found in suomilide and banyaside (Section IV.C.2) (Hanessian *et al.*, 2004). Spumigins isolated from *Nodularia spumigena* do not contain the Choi moiety, but do contain Hpla, homotyrosine (Hty), Pro or MePro, and either Arg or argininol (Fujii *et al.*, 1997a). Kasumigamide, an antialgal peptide from *Microcystis aeruginosa*, also contains phenyllactic acid (Pla) at the N-terminus but, unlike aeruginosin-like peptides, contains β-Ala, 4-amino-3-hydroxy-5-indolylpentanoic acid (Ahipa), Arg, and a C-terminal phenylSer residue (Ishida and Murakami, 2000). Ahipa is thought to derive from tryptophan (Welker and von Doehren, 2006).

2. *Hybrid Linear Peptides*

Microginins from *Microcystis* (Fig. 16) are linear *N*-acyl lipopeptides containing four to six amino acids. Nostoginins and oscillaginins share the same structural features, but are produced by *Nostoc* and *Oscillatoria*, respectively. These peptides are characterized by a modified *β*-amino acid at the N-terminus and the presence of tyrosine residues, commonly found at the C-terminus. The first reported microginin, isolated in 1993 from a strain of *Microcystis*, contains a 3-amino-2-hydroxydecanoic acyl moiety (Ahda) at the N-terminus and two C-terminal tyrosine residues (Okino *et al.*, 1993a). Microginin FR1 also contains Ahda and two terminal tyrosines flanking variable proteinogenic amino acids (Neumann *et al.*, 1997). Microginin 478 contains an *N*-methyl variant of Ahda and two C-terminal tyrosine residues (Ishida *et al.*, 2000a). Microginins 51A and -B also contain the *N*-methyl Ahda moiety and three tyrosine residues, only one of which is at the C-terminus (Ishida *et al.*, 2000a). Microginins T1 and T2 contain a dityrosine C-terminus and Ahda. However, microginin T1 contains a chlorine atom at the end of the decanoic acid chain (Kodani *et al.*, 1999). Mono- and dichlorination of the Ahda residue is a common feature of microginins. Microginins 91A-E contain variable chlorination of this N-terminal *β*-amino acid, as do microginins 299A-D, 99A and -B, and oscillaginin. Microginins 91C-E are pentapeptides with a C-terminal tyrosine residue, while variants 91A and -B are tetrapeptides lacking this tyrosine residue, terminating instead with a proline (Ishida *et al.*, 2000a). Similarly, microginins 299A-C are hexapeptides with a C-terminal tyrosine, and microginin 299D is a pentapeptide variant that lacks the C-terminal tyrosine and ends with a proline residue (Ishida *et al.*, 1997b, 1998b). A C-terminal proline residue is also found in microginins 99A and -B (Ishida *et al.*, 1998b). In oscillaginins A and B the peptide chain ends in Hty (Sano and Kaya, 1997). Nostoginins are microginin variants isolated from *Nostoc* that contain 3-amino-2-hydroxyoctanoic acid (Ahoa) at the N-terminus. Nostoginin BN578 contains a single C-terminal tyrosine, whereas nostoginin BN741 contains a dityrosine C-terminus (Ploutno and Carmeli, 2002). Microgninin SD755 has the same structure as nostoginin BN741, with the addition of a methyl group on the *β*-amino group of Ahoa (Reshef and Carmeli, 2001). Several variants of these lipopeptides show strong angiotensin-converting enzyme (ACE) and aminopeptidase M (APM) inhibition. Cyanostatins are APM inhibitors isolated from *Microcystis* that also resemble microginins. Two variants have been isolated, both of which contain an N-terminal 3-amino-2-hydroxydecanoic acid (Ahda) motif. Cyanostatin A

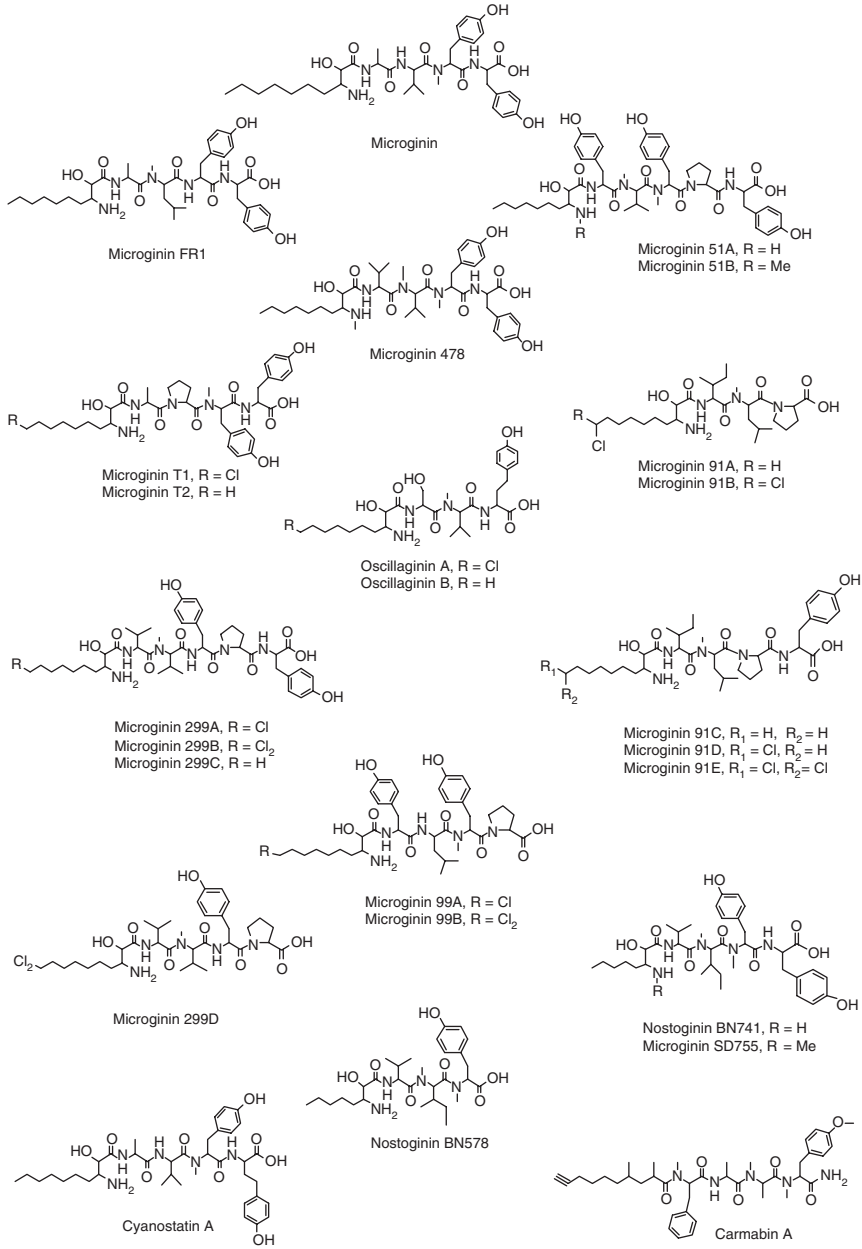


FIG. 16. Microginin variants and derivatives. Most of these hybrid linear peptides contain at least one tyrosine residue with the exception of microginins 91A and -B.

has the sequence Ahda-Ala-Val-NMeTyr-Hty (Fig. 16), whereas cyanostatin B contains Ahda-Tyr-NMeIle-Pro-Tyr (Sano *et al.*, 2005). Yet other microginin-like peptides are found in *Lyngbya* extracts.

The carmabins are lipotetrapeptides with a high proportion of *N*-methylated amino acids. Carmabins A and B differ only in the nature of the terminal functionality of the acyl moiety: The acetylenic group in carmabin A (Fig. 16) is replaced by a methyl ketone group in carmabin B (Hooper *et al.*, 1998).

The linear peptides majusculamides A, B, and D and deoxymajusculamide D from the marine species *Lyngbya majuscula* contain an *N*-octanoyl or *N*-decanoyl moiety, as well as *N*,*O*-dimethyl tyrosine and *N*-methylVal amino acids. Majusculamides A and B are nontoxic (Marner *et al.*, 1977), whereas the larger peptide majusculamide D and its deoxy derivative are cytotoxic (Fig. 17). Majusculamide D also contains hydroxyproline and a terminal pyrrolin-2-one (Moore and Entzeroth, 1988). The microcolins are immunosuppressive peptides from *Lyngbya* related to majusculamide D. These linear lipopeptides are composed of dimethyl octanoic acid, *N*-methylLeu, Val, *N*-methylVal, hydroxyPro, and pyrrolin-2-one (Koehn *et al.*, 1992).

A series of peptides, containing *N*-acyl, *N*-methyl hydrophobic amino acids such as Ala, Val, Leu, or Phe often followed by a Val residue, have been isolated from various cyanobacteria. For example, dragonamide (Fig. 17) is a cytotoxic lipopeptide isolated from *Lyngbya* containing a modified octynoic acid at the N-terminal Val (Jiménez and Scheuer, 2001). Spiroidesin is a simple lipopetide with antialgal activity produced by *Anabaena*. Spiroidesin contains an *N*-hexanoyl moiety, followed by two Hty residues and a C-terminal phenylalanine (Fig. 17) (Kaya *et al.*, 2002).

Tasiamides are cytotoxic lipopeptides from *Symploca* (Fig. 17). While they both contain *N*-acyl aliphatic residues followed by Gln. Tasiamide contains a modified valeric acyl group at the N-terminus (2-hydroxy-3-methylvaleric acid) (Williams *et al.*, 2002a), whereas tasiamide B contains a lactic acyl group at the N-terminus as well as an internal modified pentanoic acid (4-amino-3-hydroxy-5-phenylpentanoic acid), possibly derived from Phe (Fig. 17) (Williams *et al.*, 2003d).

Suomilide, a glycosidic peptide from *Nodularia*, contains an azabicyclononane moiety determined to be 4-amino-5,7,9-trihydroxy-2-azabicyclononane-3-carboxylic acid (Abn). Other components of suomilide include *allo*-Ile, 2-*O*-methylglyceric acid 3-*O*-sulfate (Mgs), 1-amidino-3-(2-aminoethyl)-3-pyrroline (Aaep), and a glucose sugar with two pendant hexanoic acids (Fig. 17) (Fujii *et al.*, 1997b). The Aaep residue is also found in the aeruginosin derivative oscillarin and

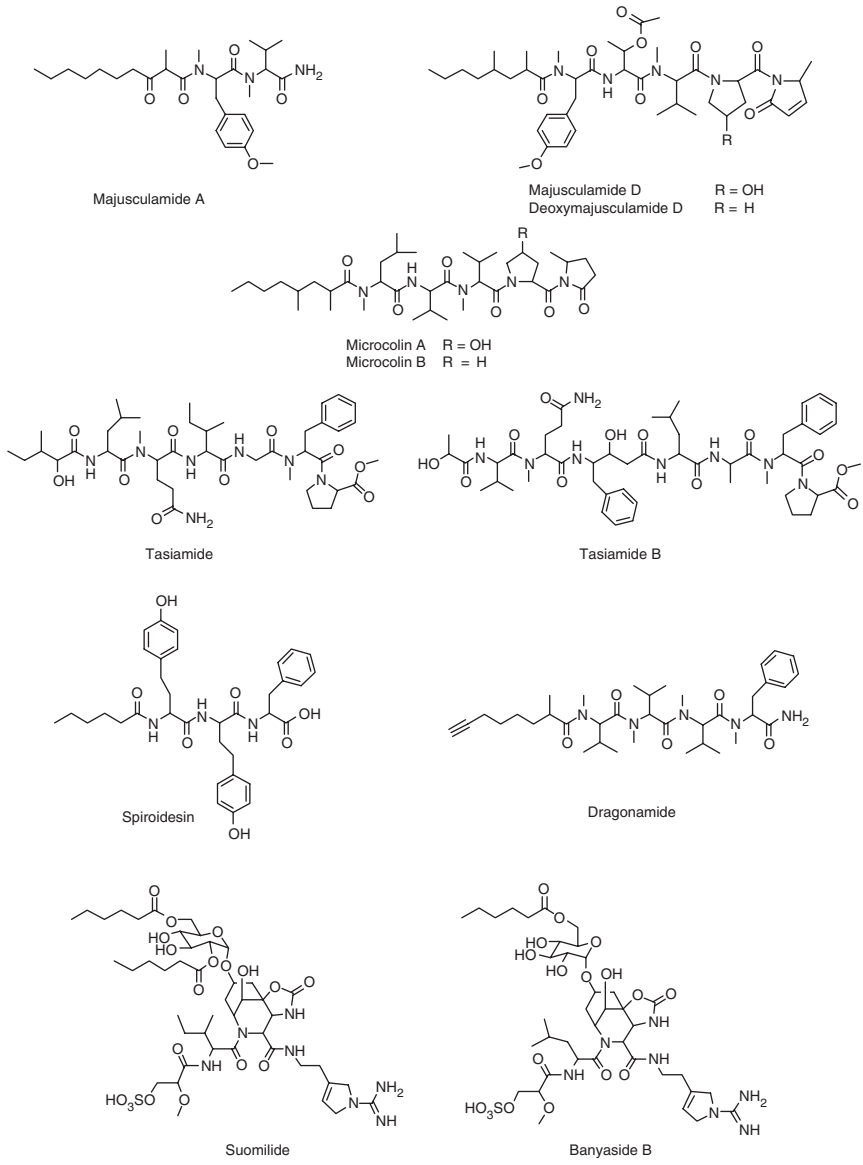


FIG. 17. Structural representatives of hybrid linear peptides isolated from cyanobacteria. All of these peptides contain an *N*-acyl group, some of which arise from α-hydroxy acids.

the Mgs residue is also found in the cyclic metabolite oscillapeptin (Section IV.E.1).

Banyasides A and B (Fig. 17), serine protease inhibitors isolated from *Nostoc*, are structurally similar to suomilide, but contain Leu in place of *allo*-Ile and only a single hexanoic acid on the glucose residue. Banyasides A and B differ in the placement of the hexanoic acid on the glucose unit. Like the Choi moiety of the aeruginosins, the Abn residue of suomilide and banyasides is speculated to be derived from the aromatic amino acid tyrosine. However, Choi biosynthesis is thought to be initiated by reduction of the phenol ring to the cyclohexenol, whereas Abn biogenesis probably begins with the oxidation of tyrosine to the quinone methide. Such an oxidation is proposed as the initial step in the biosynthesis of radiosumin (Pluotno and Carmeli, 2005).

3. *Linear Peptides Containing Thiazole/Oxazole Rings*

All of the compounds described in this section contain thiazole or oxazole rings, the biosynthesis of which is described in Section II.B.I. Tantazoles and mirabazoles are an interesting group of oxidized tetra- and pentapeptides with tumor selective cytotoxic activity from *Scytonema mirabile* (Fig. 18). Both groups of metabolites are notable in that they contain *C*-methyl groups at the α-carbon of each amino acid. Such hypermethylation of peptides is rare in nature. Mirabazoles contain four contiguous cysteine-derived thiazole rings and a pendant isopropyl group at C2 of the first thiazole ring, which may arise through incorporation of isobutyrate. Tantazoles also contain four cysteine-derived thiazole rings, as well as a C-terminal threonine-derived oxazole ring containing an *N*-methyl amide group (Carmeli *et al.*, 1990c, 1991a, 1993b). A C-terminal thiazole ring occurs in aeruginosamide, a small linear peptide from *Microcystis aeruginosa*, and in this case the carboxyl group is methylated, while the N-terminal residue is doubly prenylated (Lawton *et al.*, 1999). In the case of muscuride A from *Nostoc muscorum*, the first cyanobacterial metabolite containing *N*-(2-methyl-3-buten-2-yl) valine, the terminal threonine oxazole residue is found as a prenyl ester (Nagatsu *et al.*, 1995).

Anachelins are siderophores isolated from *Anabaena cylindrica*. These iron-chelating agents possess a 2-hydroxyphenyl-oxazoline system, a structural feature similar to the mycobactins, a family of siderophores produced by *Mycobacterium tuberculosis* (De Voss *et al.*, 2000). Further structural complexity in the anachelins is provided by the unique residue 1,1-dimethyl-3-amino-1,2,3,4-tetrahydro-7,8-dihydroxy-quinolin (Dmaq) (Fig. 18) (Itou *et al.*, 2001).

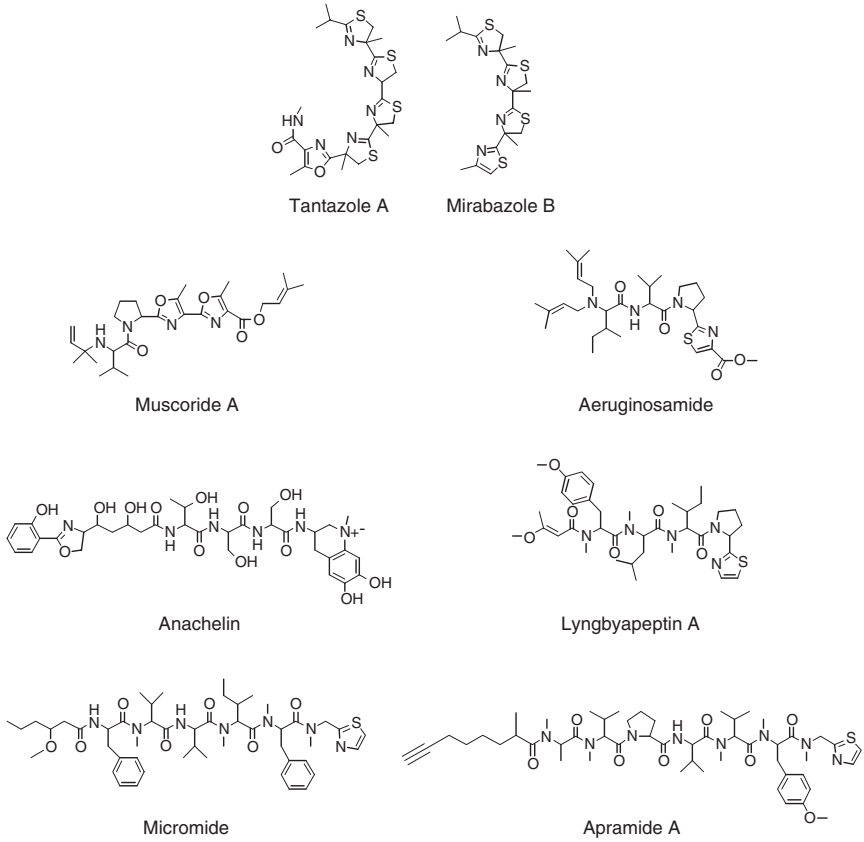


FIG. 18. Thiazole and oxazole containing linear cyanopeptides. The tantazoles and mirabazoles are hypermethylated. In lyngbyapeptin A, micromide, and apramide A, the carboxyl group of the terminal cysteine has been lost.

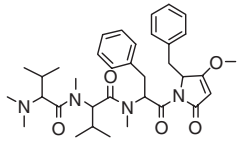
Lyngbyapeptins, isolated from *Lyngbya*, are further examples of thiazole-containing lipopeptides. A notable feature of these compounds is the rare 3-methoxy-2-butenoyl moiety. Lyngbyapeptins also demonstrate several features found in other *Lyngbya* metabolites such as a high level of *N*-methylation and an *N,O*-dimethyl tyrosine residue (Fig. 18) (Klein *et al.*, 1999a).

Apramides from *Lyngbya* and micromides from *Symploca* show strong structural homology, sharing a thiazole ring and a modified alkyl chain (Fig. 18). Apramides contain a modified octenoic or octynoic acid (Luesch *et al.*, 2000a), whereas the cytotoxin micromide contains a 3-methoxyhexanoyl moiety. Despite their structural homology, apramides are more cytotoxic than micromide, with IC_{50} values several

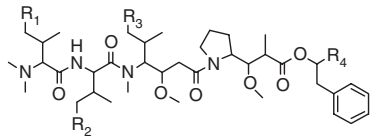
orders of magnitude lower (Williams *et al.*, 2004). This difference in activity could be attributed to the presence of a Pro residue in the chain which would be expected to confer a considerably different conformation on the molecule.

4. *Linear Dolastatins and Derivatives*

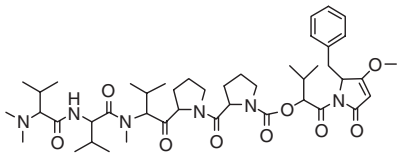
Dolastatins are a large group of cytotoxic cyanopeptides originally isolated from the sea hare *Dolabella auricularia*. Recently, several dolastatins and dolastatin analogues have been isolated from cyanobacterial species, providing evidence that cyanobacteria are the true biological source of dolastatins (Fig. 19). The reader is directed to a more exhaustive review on the cyanobacterial origin of dolastatins and other anticancer agents originally isolated from macroorganisms (Luesch *et al.*, 2002a). Dolastatins define five classes of metabolites with large structural diversity. The first class of dolastatins is composed of linear peptides and is discussed in this section. The remaining four are cyclic and will be discussed later with other cyclic peptides of the same structural class. Linear dolastatins are characterized by a series of aliphatic amino acids followed by one or two proline residues. Most of the amide nitrogens are methylated and a distinctive residue of variable structure is at or near the C-terminus. This distinctive residue can be a terminal thiazole-phenethylamine referred to as dolaphenine (Doe), a pyrrolidone ring, a terminal primary amide, or a ketide-extended amino acid. The only linear dolastatin isolated from a cyanobacterial species (*Symploca*) is dolastatin 10 (Luesch *et al.*, 2001a). Dolastatin 10 is an exceptionally potent antitubulin agent with demonstrated anticancer activity and is currently in Phase II clinical trials. Symplostatins are dolastatin analogues isolated from *Symploca*, also encompassing both linear and cyclic structures. Linear symplostatins will be discussed in this section and cyclic symplostatins will be discussed in Section IV.E.1. The linear peptide symplostatin 1 is a dolastatin 10 analogue, differing only in the N-terminus being *N,N*-dimethyl Ile instead of *N,N*-dimethylVal (dolavaline, Dov) (Harrigan *et al.*, 1998a). Symplostatin 3, an isodolastatin H analogue, differs from dolastatin 10 only in the C-terminal residue (Luesch *et al.*, 2002f). Malevamide D, also isolated from *Symploca* sp., is a highly cytotoxic peptide ester that shares strong structural homology with symplostatin 3 and isodolastatin H (Horgen *et al.*, 2002). Belamide is a highly methylated tetrapeptide also isolated from *Symploca* sp. that exhibits cytotoxic activity via tubulin-destabilizing antimitotic activity. Belamide is a small tetrapeptide that, like dolastatin 15, has a terminal pyrrolidone moiety as well as a series of highly methylated aliphatic amino acids (Simmons *et al.*, 2006).



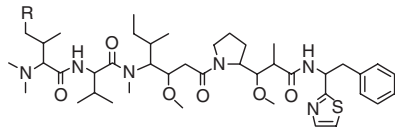
Belamide



Symplostatin 3: $R_1 = H$ $R_2 = H$ $R_3 = CH_3$ $R_4 = CO_2H$
Malevamide D: $R_1 = CH_3$ $R_2 = H$ $R_3 = H$ $R_4 = CH_2OH$
Isodolastatin H: $R_1 = H$ $R_2 = CH_3$ $R_3 = CH_3$ $R_4 = CH_2OH$



Dolastatin 15



Symplostatin 1 R = CH_3
Dolastatin-10 R = H

FIG. 19. Linear dolastatins and analogues isolated from cyanobacteria. These peptides are characterized by a high degree of *N*-methylation and a distinctive C-terminal residue of variable structure.

D. CYCLIC PEPTIDES

1. *Simple Cyclic Peptides*

Nostocyclamide, microcyclamide, tenuecyclamide, banyascyclamide, westiellamide, raocyclamides, and dendroamides are cyclic hexapeptides in which the ring system contains amide links only (Fig. 20). All contain three heteroaromatic rings (thiazoles and oxazoles) symmetrically arranged in the peptide chain with different connecting aliphatic amino acids providing structural diversity. This structural class was first described from the ascidian *Lissoclinum* with the discovery of bistratamides (Degnan *et al.*, 1989), which are most likely produced by cyanobacterial symbionts. Nostocyclamides reported from *Nostoc* contain two thiazole rings and one methyl oxazole and are connected by Gly, Ala, and Val residues (Jüttner *et al.*, 2001; Todorova *et al.*, 1995). Microcyclamide from *Microcystis* also contains two thiazole and one methyl oxazole rings and contains Ala, Ile, and N^{δ}-methyl His as connecting residues (Ishida *et al.*, 2000b). Tenuecyclamide C from *Nostoc* and dendroamide B from *Stigonema* are almost identical and contain methionine as one of the

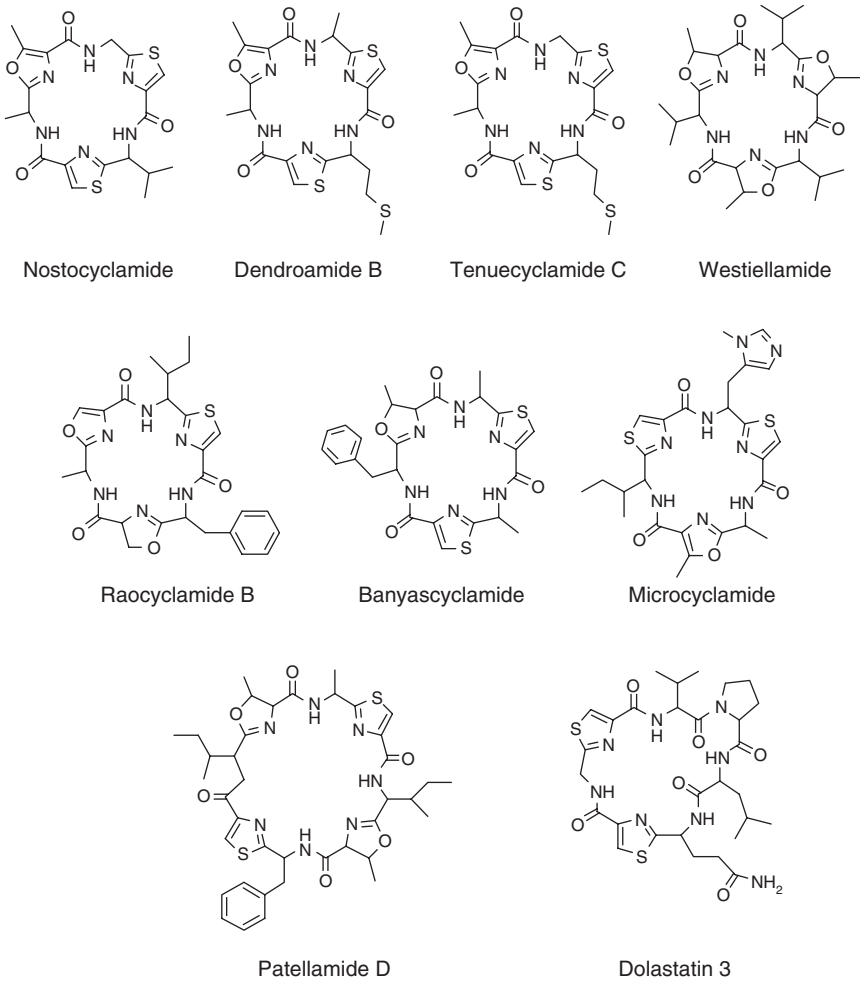


FIG. 20. Cyclamide derivatives from cyanobacteria. Interestingly, the patellamides from *Lissoclinum* are produced ribosomally.

connecting amino acids (Banker and Carmeli, 1998; Ogino *et al.*, 1996). Raocyclamide B from *Oscillatoria* (Admi *et al.*, 1996) and banyascyclamide from *Nostoc* (Ploutno and Carmeli, 2002) both contain a Phe residue. The symmetrical westiellamide from *Westiellopsis* contains three methyl oxazoline rings and three Val residues (Prinsep *et al.*, 1992b). Patellamides (Fig. 20), first isolated from *Lissoclinum* and later reported from the cyanobacterium *Prochloron*, share many of the structural features of the

previously described compounds but contain four heteroaromatic rings (McDonald and Ireland, 1992; Schmidt *et al.*, 2005). Perhaps surprisingly, genetic analysis has revealed that patellamides are derived from ribosomally encoded genes and their function has been further confirmed by heterologous expression in *E. coli* (Schmidt *et al.*, 2005). A few dolastatin analogues also contain this general structure, including dolastatin 3, E, and I. Dolastatin 3, originally isolated from the sea hare *Dolabella* and described from *Lyngbya*, exhibits HIV-1 integrase inhibition (Fig. 20) (Mitchell *et al.*, 2000; Pettit *et al.*, 1982).

Kororamide, a cyclic nonapeptide containing thiazoline, thiazole, and a proline ring, is an HIV-1 integrase inhibitor isolated from *Lyngbya* (Fig. 21) (Mitchell *et al.*, 2000). Wewakazole is another cyclic peptide from *Lyngbya* (Fig. 21). This dodecapeptide contains three oxazoline rings (one of which is probably derived from threonine) and three proline residues (Nogle *et al.*, 2003).

In contrast to the peptides just described, the cyclic nonapeptide oscillacyclin isolated from *Planktothrix* (originally *Oscillatoria*) is composed only of protein amino acids (Fig. 21) (Fujii *et al.*, 2000). Agardhipeptins are another group of cyclic hepta- and octapeptides isolated from *Oscillatoria agardhii* containing only protein amino acids. Agardhipeptin A (Fig. 21) has a structure of cyclo-(His-Gly-Trp-Pro-Trp-Gly-Leu) and agardhipeptin B has a structure of cyclo-(Trp-Leu-Pro-Trp-Ala-Pro-Trp-Val). Only agardhipeptin A possesses plasmin inhibitory activity (Shin *et al.*, 1996a).

Nostocyclopeptides, the cyclic heptapeptides from a *Nostoc* sp., contain a unique imino linkage and a *C*-methylproline residue (Fig. 21). Biosynthetic studies have revealed that this *C*-methylproline group originates from a leucine precursor (Luesch *et al.*, 2003). Two variants, A1 and A2 have been described, both of which exhibit weak cytotoxic activity (Golakoti *et al.*, 2001). Nostocyclopeptide A2 differs from A1 by the presence of a Phe residue in place of Leu separating the *C*-methylproline and imino-linked tyrosine. Molecular genetic studies have shown that two NRPS genes in the first operon assemble the peptide (Tyr-Gly-DGln-Ile-Ser-MePro-Leu/Phe)-S-NcpB and a unique terminal reaction results in reduction of the terminal carboxyl function to a linear aldehyde that undergoes intermolecular condensation with the amino group of the N-terminus tyrosine to form a stable imine bond (Becker *et al.*, 2004).

Oscillatorin (Fig. 21), a chymotrypsin inhibitor isolated from *Oscillatoria*, is a cyclic decapeptide containing protein amino acids and oscillatoric acid (1,2,3,3a,8,8a-hexahydro-3a,9-methyl-2-butenyl)-pyrroloindol-2-carboxylic acid (Osc), a unique amino acid that has only

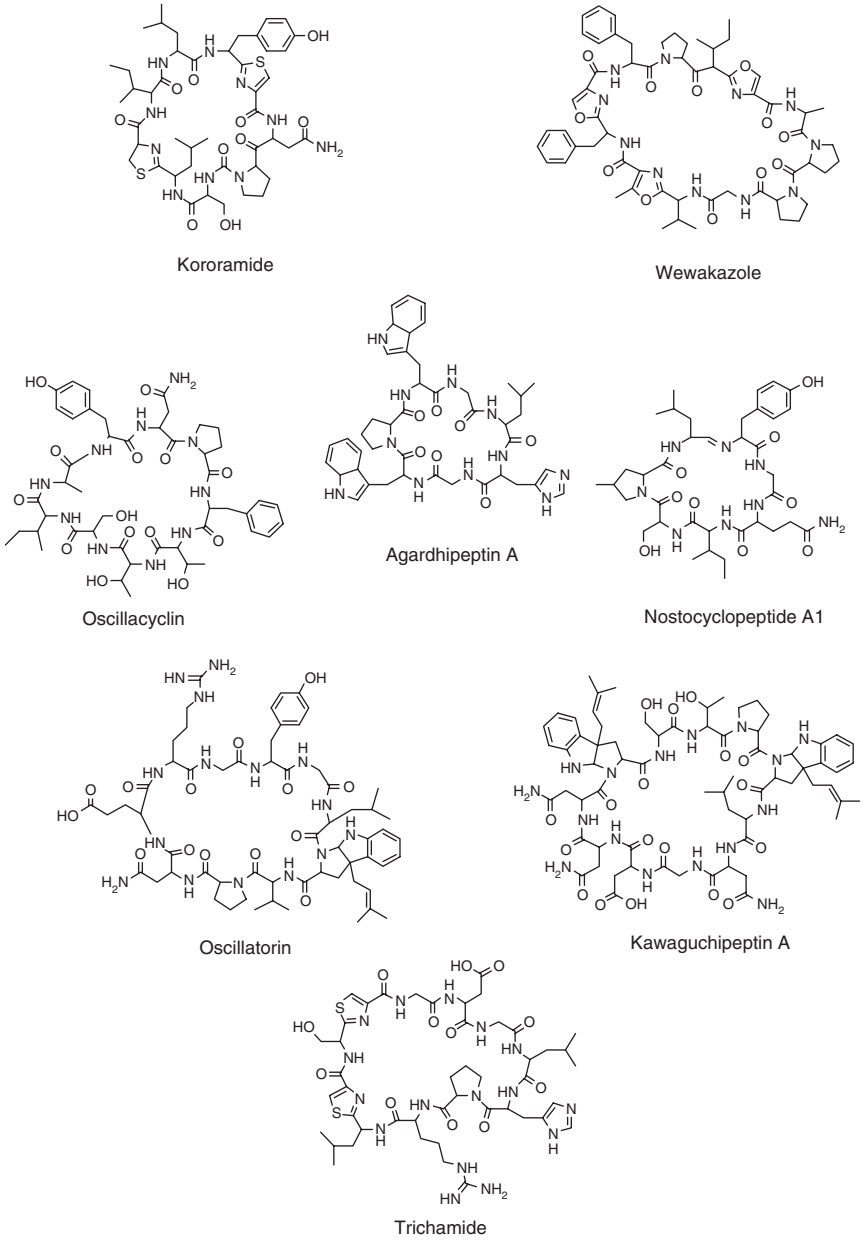


FIG. 21. Simple cyclic peptides from cyanobacteria. Oscillatorin and kawaguchipeptins contain prenylated tryptophan residues while nostocyclopeptolide has a unique imino-linked tyrosine. The structure of trichamide has been predicted from the genome sequence.

been reported in oscillatorin. The biosynthesis of Osc is thought to involve an intramolecular cyclization of tryptophan with concomitant addition of an isoprene unit (Radau, 2000; Sano and Kaya, 1996b). Prenylation of tryptophan residues is also a feature of the kawaguchipeptins (Fig. 21), a family of antibacterial cyclic undecapeptides isolated from *Microcystis* (Ishida *et al.*, 1996, 1997a).

Trichamide is a cyclic peptide from *Trichodesmium* for which the structure was predicted from the genome sequence and confirmed by mass spectrometry. This undecapeptide contains two cysteine-derived thiazoles and is cyclized by an N–C-terminal amide bond (Fig. 21) (Sudek *et al.*, 2006).

2. *Cyclic Hybrid Peptides Containing* β*-Amino Acids*

Cyclic hybrid peptides are assembled from PKS- and NRPS-derived biosynthetic components. All of the cyclic hybrid peptides described in this section incorporate the polyketide-derived component in the cyclic ring system. The most studied of these are the microcystins, a large family of heptapeptide hepatotoxins with the generalized structure cyclo-(Ala-X-MeAsp-Y-Adda-Glu-Mdha) (Fig. 22). Adda is the unique C_{20} β-amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid. MeAsp is methyl aspartic acid and Mdha is *N*-methyldehydroalanine. Substitutions at position 2 and 4 (X and Y) give rise to more than 20 primary microcystin analogues, with alterations in other constituent amino acids resulting in more than 90 reported microcystins to date. The most common and most toxic microcystin, microcystin-LR (Fig. 22), contains Leu (L) at position 2 and Arg (R) at position 4. Microcystins have been isolated from *Microcystis*, *Anabaena*, *Nostoc*, *Planktothrix*, and *Aphanizomenon* (Zurawell *et al.*, 2005).

The toxic effects of microcystins are due to their inhibition of eukaryotic protein phosphatases 1 and 2a (PP1 and PP2a). PP1 and PP2a are vital in the control of several cellular processes, including carbohydrate metabolism, muscle contraction, and cell division. Microcystins cannot cross cell membranes, but are actively transported by the bile acid transporter mechanism, providing a vector for entry into the liver where they can cause acute liver failure and tumor promotion (Chorus *et al.*, 2000; Lawton and Codd, 1991).

Microcystin-LR was the first cyanobacterial metabolite whose nonribosomal origin was confirmed by knockout mutagenesis (Dittmann *et al.*, 2001a). Feeding experiments have revealed the activation and incorporation of both amino acid and acetate units into microcystins. The microcystin synthetase (mcy) gene cluster was found to comprise

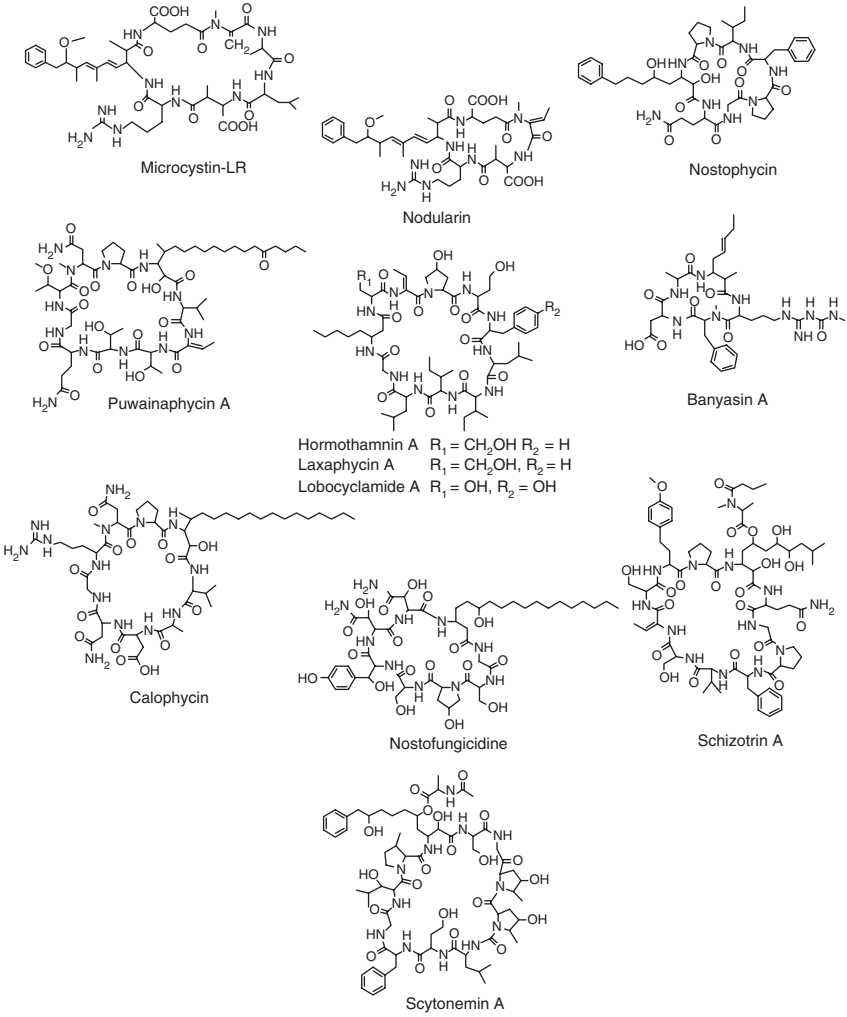


FIG. 22. Cyclic hybrid peptides containing β-amino acids. Although schizotrin A and scytonemin A contain ester linkages, these compounds are grouped with the peptides because the ester linkages are not part of the macrocyclic ring.

10 genes, encoding peptide synthetases, polyketide synthases, and tailoring functions (Tillett *et al.*, 2000). The integration of a β-amino acyl moiety in the ring of cyclic peptides is a recurring theme in the formation of many cyclic peptides. For example, the Adda unit is found in nodularin (Fig. 22), a cyclic pentapeptide from *Nodularia* sp. which also

inhibits PP1 and PP2A. Similarly nostophycin, isolated from *Nostoc* sp., incorporates the unique *β*-amino acid 3-amino-2,5-dihydroxy-8-phenyloctanoic acid (Ahoa) in the ring structure, and is thought to be biosynthetically related to the microcystins (Fig. 22) (Dembitsky and Rezanka, 2005; Fujii *et al.*, 1999).

A series of cyclic peptides have been discovered that incorporate a short (C_8) aliphatic *β*-amino acyl moiety in the formation of their respective ring systems (Fig. 22). These include the antifungal laxaphycins from *Anabaena* (Frankmolle *et al.*, 1992a,b), hormothaminin, a cyclic undecapeptide from *Hormothamnion* (Gerwick *et al.*, 1989, 1992), and the lobocyclamides, another group of antifungal agents that contain either 3-aminooctanoic acid (Aoc) or 3-aminodecanoic acid (Ade) (MacMillan *et al.*, 2002). In the case of banyasin A isolated from *Nostoc*, the C_8 acyl chain has undergone further elaboration through methylation and introduction of a double bond. Banyasin also contains the previously unreported *N*-methylcarboxyamide arginine (Pluotno and Carmeli, 2005).

The puwainaphycins, calophycin, and nostofungicidine represent a series of cyclic peptides which incorporate larger, more complex, *β*-amino acyl units in the construction of the ring system (Fig. 22). Puwainaphycin A, a cyclic decapeptide from *Anabaena*, contains a 3-amino-2-hydroxy-4-methyl-14-oxostearyl (Ahmos) residue, as well as four threonine-derived amino acids, two of which undergo further modification through dehydration or methylation. Puwainaphycin C, a halogenated variant in which the carbonyl at C14 of the stearic acid chain is replaced by chlorine, is a potent cardioactive agent (Gregson *et al.*, 1992; Moore *et al.*, 1989a). Calophycin, a broad spectrum antifungal agent isolated from *Calothrix* (Moon *et al.*, 1992), contains a related C_{18} *β*-amino acyl moiety similar to that found in the puwainaphycins, as does nostofungicidine, an antifungal cyclic lipopeptide from *Nostoc* which contains the novel 3-amino-6-hydroxystearic acid (Ahs) group (Kajiyama *et al.*, 1998).

The macrocyclic ring of schizotrin A, a cyclic undecapeptide from *Schizothrix* (Pergament and Carmeli, 1994), is constructed entirely with amide bonds. The only ester link resides in the heavily functionalized acyl side chain (3-amino-2,7,8-trihydroxy-10-methyl-5-oxydecanoic acid), which is esterified with an *N*-methyl-*N*-butanoyl alanine moiety (Fig. 22). Scytonemin A, a cyclic depsipeptide calcium antagonist from *Scytonema* (Helms *et al.*, 1988) follows a similar biogenetic pattern. An acyl unit, identified as the *β*-amino acid 3-amino-2,5,9-trihydroxy-10-phenyldecanoic acid (Ahda), and esterified in this case with an

N-acetylalanine unit, provides the starter acyl unit for the macrocyclic peptide ring formed entirely with amide bonds (Fig. 22).

3. *Cyclic Peptides with a Uriedo Linkage*

Anabaenapeptins A-J have been isolated from *Anabaena*, *Nodularia*, *Planktothrix*, *Oscillatoria*, and *Aphanizomenon*. The ring system of this family of cyclic pentapeptides arises by formation of an amide bond between the side chain amine of lysine and the carboxyl carbon of an amino acid four residues removed. Further novelty results from the uriedo-linked variable amino acid and several variants are potent carboxypeptidase inhibitors. Anabaenopeptin A (Fig. 23) is composed of Lys-Val-Hty-*N*-methylAla-Phe with a uriedo-linked Tyr. In anabaenopeptin B the Tyr is replaced with Arg. Most anabaenopeptins contain this general structure, usually containing Tyr or Arg as the uriedo-linked amino acid, with the exception of anabaenopeptins I and J which contain Ile, C which contains Lys, and D which has a uriedo-linked Phe residue. Other variations come from substitutions at Phe (Ile in anabaenopeptin H and Leu in anabaenopeptin I) as well as *N*-methylAla (*N*-methylHty in anabaenopeptin G) (Fujii *et al.*, 1997a; Harada *et al.*, 1995; Itou *et al.*, 1999b; Murakami *et al.*, 1997b, 2000; Shin *et al.*, 1997c). The ferintoic acids are anabaenopeptin-like cyclic peptides from *Microcystis aeruginosa*. In the case of ferintoic acid A (Fig. 23), the only difference is that the tyrosine group in anabaenopeptin A is replaced by tryptophan (Williams *et al.*, 1996). Ferintoic acid B has the same structure as ferintoic acid A; however, it contains Ile instead of Val.

Another group of anabaenopeptin derivatives include the protein phosphatase inhibitors oscillamides B and C from *Planktothrix* and the chymotrypsin inhibitor oscillamide Y (Fig. 23) from *Oscillatoria*. Oscillamide B differs from oscillamide Y in that Met replaces Ile and the uriedo-linked amino acid is an Arg; oscillamide C also contains a uriedo-linked Arg but retains the Ile and contains *N*-methylHty in place of *N*-methylAla. The protein phosphatase inhibitory activity of oscillamides B and C is believed to proceed by a different mechanism than that of the microcystins and nodularin, and may be dependent on the presence of the Arg and *N*-methylHty residues (Sano and Kaya, 1995; Sano *et al.*, 2001).

Nodulapeptins, structural homologues to anabaenopeptins, have been reported from *Nodularia*. Nodulapeptins contain a uriedo-linked Ile and several distinctive residues, including homophenylalanine, *N*-methylHty, an *O*-acetylSer residue, and either methionine sulfoxide (B) or methionine sulfone (A) (Fig. 23) (Fujii *et al.*, 1997a). Like the

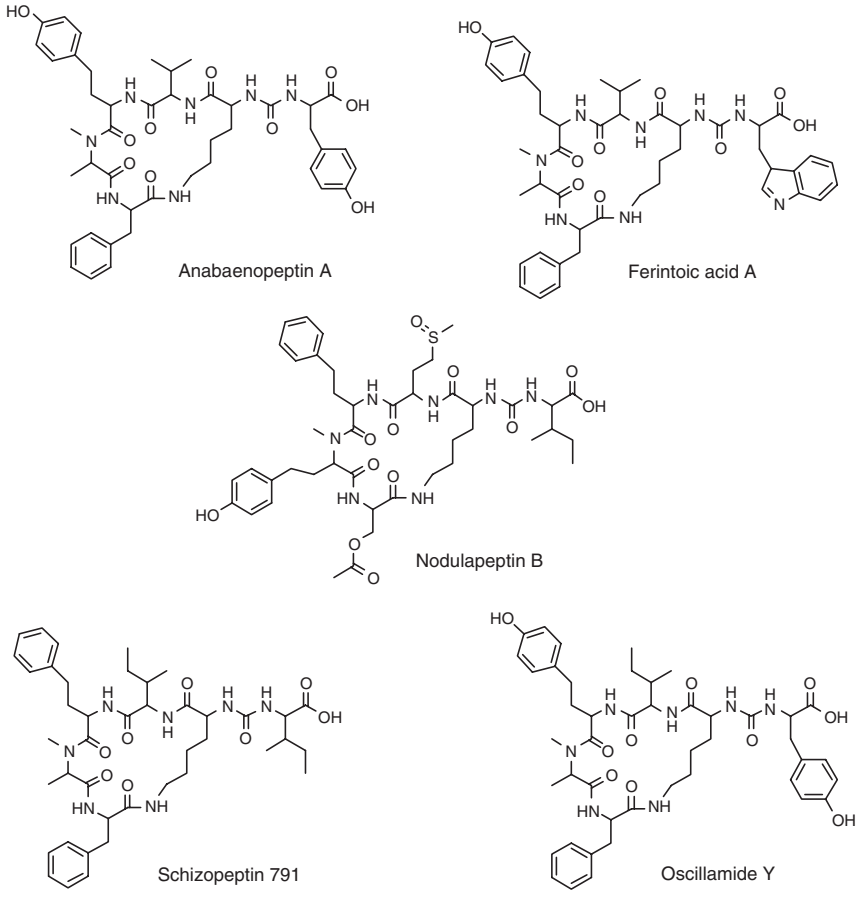


FIG. 23. Cyclic cyanopeptides containing a uriedo-linked variable side chain.

nodulapeptins, schizopeptin 791 (Fig. 23), a trypsin inhibitor isolated from *Schizothrix*, contains a terminal uriedo-linked isoleucine residue, as well as homophenylalanine (Reshef and Carmeli, 2002).

E. CYCLIC DEPSIPEPTIDES

Cyclodepsipeptides are thought to be nonribosomally synthesized with the termination of biosynthesis occurring by lactonization with a terminal or side chain hydroxyl group resulting in a macrocyclic lactone or branched lactone structure.

1. *Cyclic Depsipeptides with Ahp*

Numerous cyclic depsipeptides have been isolated from cyanobacteria containing the unique residue 3-amino-6-hydroxy-2-piperidone referred to as Ahp (Weckesser *et al.*, 1996). These cyclic Ahp-containing depsipeptides contain a ring closure with an ester bond between the β-hydroxy group of threonine and the carboxy-terminus of the C-terminal amino acid of a proposed linear precursor. The threonine residue required for lactone ring formation is further elaborated by addition of a series of amino acids and *N*-acyl groups, including modified hydroxy acids and nonamino acid constituents such as sulfated glyceric acid residues.

The biosynthetic origin of the Ahp moiety is initiated by formylation of a Gln residue carried out by a formyl transferase domain present in the biosynthetic gene cluster (Rouhiainen *et al.*, 2000). Biosynthetic and molecular studies of the Ahp-containing anabaenopeptolides have revealed seven NRPS modules distributed across three genes (*apdA*, *apdB*, and *apdD*) (Dittmann *et al.*, 2001b). Several names have been noted in the text for these homologous structures, each will be noted but not all discussed in depth. A review of cyanopeptolin metabolites has been published (Weckesser *et al.*, 1996). Members of this cyanopeptide class are grouped according to their side chain constituents and only a few representatives of each side chain constituent are shown (Fig. 24).

Numerous cyanopeptolin derivatives contain side chains composed of proteinogenic amino acids with *N*-formylation or *N*-acylation. *N*-formyl variants include anabaenopeptolides 90A-B and 202A-B (Fujii *et al.*, 2002) and *N*-acetyl variants include oscillapeptilide 97A-B and micropeptin 88Y (Fujii *et al.*, 2000; Yamaki *et al.*, 2005). Other derivatives with *N*-acyl side chains contain C_4–C_8 fatty acid chains usually attached through glutamic acid, aspartic acid, or a glutamine residue and include cyanopeptolins A-D, cyanopeptolin 963A, numerous micropeptin variants (A, B, 88B-E, 103, 88N, 88Y), scyptolins, somamides, symplostatin 2, tasipeptins, and hofmannolin (Bister *et al.*, 2004; Ishida *et al.*, 1998a; Martin *et al.*, 1993; Matern *et al.*, 2001; Murakami *et al.*, 1997a; Nogle *et al.*, 2001; Okino *et al.*, 1993b; Williams *et al.*, 2003c; Yamaki *et al.*, 2005). An unusual example is hofmannolin, which possesses a side chain containing a 2-hydroxy-3-methylvaleric acid moiety linked through glutamic acid (Matern *et al.*, 2003).

Other cyanopeptolin variants contain Hpla as a side chain constituent. Aeruginopeptins 228A and -B and 917SA-C, as well as microcystilide contain Hpla linked to the macrocycle via Gln (Harada *et al.*, 1993, 2001; Tsukamoto *et al.*, 1993). Aeruginopeptins 95A and -B contain

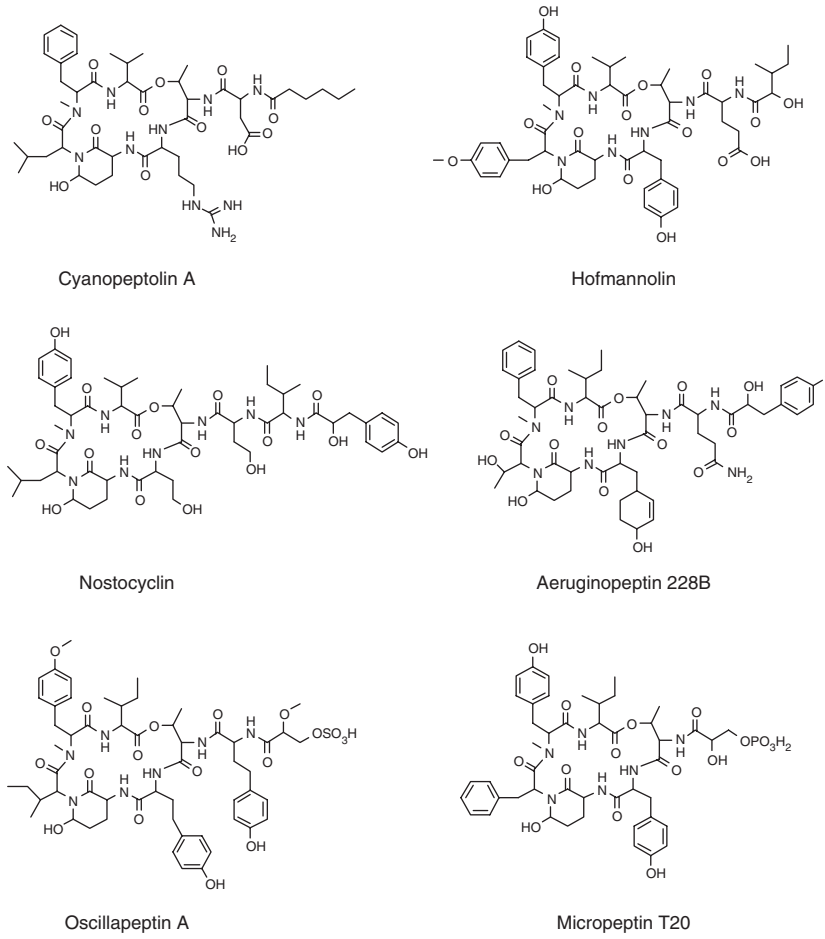


FIG. 24. Structural representatives of cyclic depsipeptides containing the unique residue Ahp. These derivatives can contain side chains composed of a fatty acid moiety, Hpla, or a modified glyceric acid residue.

Hpla and Thr linked through Gln, and the side chain of nostocyclin contains Hpla, Ile, and homoserine (Hse) (Harada *et al.*, 1993; Kaya *et al.*, 1996). Aeruginopeptin 95B, 228B, and 917S-B contain an unusual tetrahydrotyrosine (ThTyr) in the ring.

The last group of cyanopeptolin variants contain side chains composed of a modified glyceric acid constituent, usually linked to the macrocycle by proteinogenic amino acids. Micropeptin A, micropeptin 90,

cyanopeptolin S, A90720A, and oscillapeptin J contain sulfated glyceric acid and micropeptin B contains disulfated glyceric acid (Blom *et al.*, 2003; Bonjouklian *et al.*, 1996; Jakobi *et al.*, 1995; Okino *et al.*, 1993b; Yamaki *et al.*, 2005). Other glyceric acid derivatives include Mgs and 2-*O*-methylglyceric acid (Mga) found in oscillapeptins A-F, unmodified glyceric acid found in oscillapeptin G and planktopeptins BL1125 and BL1061, glutamic acid-γ-lactam found in planktopeptin BL843, and the unique glyceric acid phosphate found in micropeptin T20 (Grach-Pogrebinsky *et al.*, 2003; Itou *et al.*, 1999a; Okano *et al.*, 1999; Sano and Kaya, 1996a).

2. *Cyclic Hybrid Depsipeptides*

Hapalosin, isolated from *Hapalosiphon*, exhibits multidrug resistance reversing activity in tumor cells (Fig. 25) (Stratmann *et al.*, 1994b). The biogenesis appears unusual: a polyketide-derived C_{10} acyl chain is condensed with an α-hydroxy valeric acid unit, which in turn forms an amide link with a phenylalanine derivative, and is then subsequently extended with another acetate unit before cyclization. The cytotoxins lyngbyabellins A-C (Luesch *et al.*, 2000b,c, 2002d; Milligan *et al.*, 2000b) and hectochlorin (Marquez *et al.*, 2002) both are obtained from *Lyngbya* spp. and contain dichloro-3-hydroxy acyl chains with one or two pendant methyl groups (Fig. 25). In both cases, the acyl unit forms an ester link with an α-hydroxy acid, which is presumably transferred to an NRPS protein and extended with various amino acids before cyclization. The mechanism of chlorine introduction is interesting, since it requires addition to the carboxyl-derived (electrophilic) carbon of the starter acetate group, unlike the more common addition to an aryl ring system.

Apratoxin A (Fig. 25) is the most potent cytotoxin produced by *Lyngbya*. This unique hybrid molecule contains a thiazoline ring, linked via a putative methyl-ketide unit to the next amino acid which is an *N,O*-dimethyl tyrosine residue. The novel polyketide portion, 3,7-dihydroxy-2,5,8,8-tetramethylnonanoic acid (Dtena), is proposed to originate from a starter unit containing a *tert*-butyl moiety (Luesch *et al.*, 2001c, 2002e).

In the case of antillatoxin (Fig. 25), an exceptionally potent ichthyotoxin from *Lyngbya* (Orjala *et al.*, 1995b), a more complex polyketide δ-hydroxy acyl chain (5-hydroxy-4,6,8,10,10-pentamethyl-3-methyleneundeca-6,8-dienoic acid), is presumably transferred to an NRPS protein to permit the addition of Ala, *N*-methylVal, and Gly. Like apratoxin, the *tert*-butyl group found in the polyketide portion is most likely part of the starter unit.

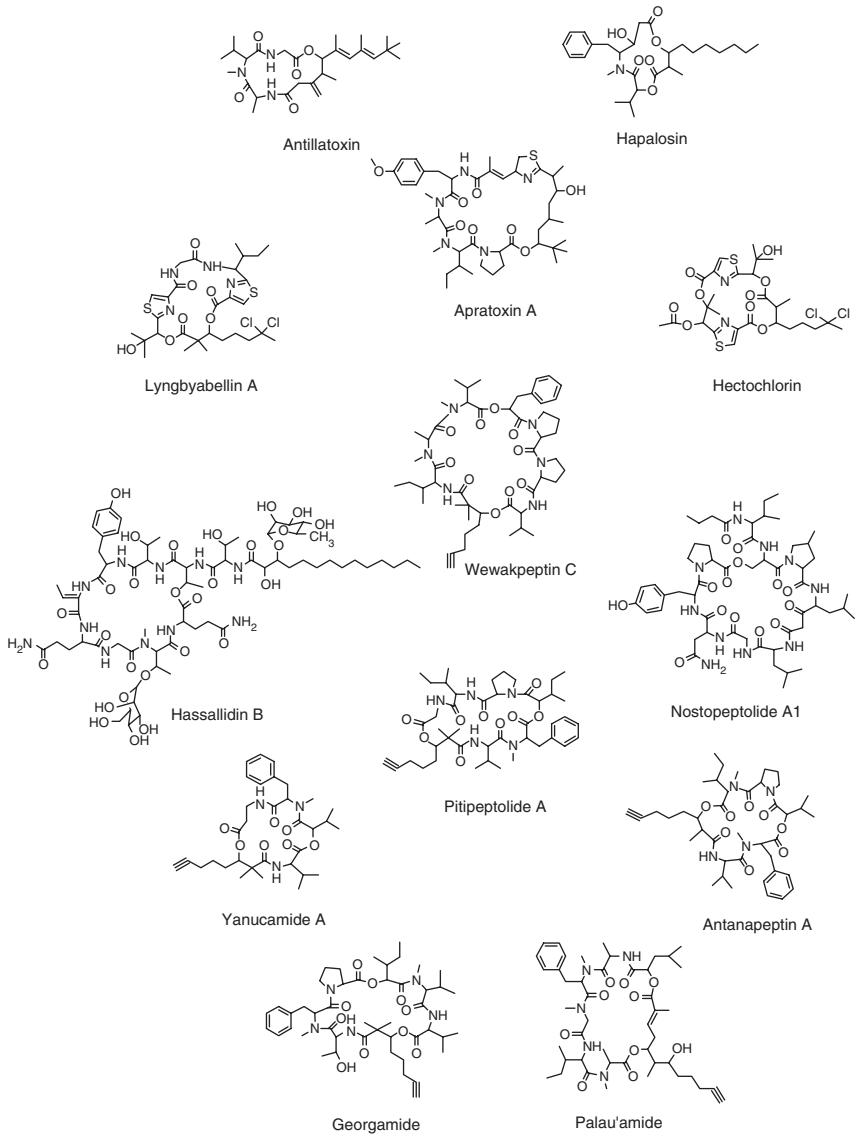


FIG. 25. Cyclic hybrid depsipeptides isolated from cyanobacteria.

Several acetylenic depsipeptides have been reported from *Lyngbya* sp. The yanucamides (Sitachitta *et al.*, 2000b) were isolated from a *Lyngbya/Schizothrix* assemblage, while the antanapeptins (Nogle and Gerwick,

2002b), pitipeptolides (Luesch *et al.*, 2001b), georgamide (Wan and Erickson, 2001), and wewakpeptins (Han *et al.*, 2005a) were isolated from *Lyngbya* spp. (Fig. 25). All of these cyclic depsipeptides arise through a biogenetic pathway involving a 2-alkyl-3-hydroxy acyl chain often containing an acetylenic functional group, and which forms an amide link with Val (yanucamides, antanapeptins, and pitipeptolides), Ile (wewakpeptin), or Thr (georgamide) before further extension with other amino acids and eventual cyclization. Another acetylenic depsipeptide, palau'amide (Williams *et al.*, 2003e), contains a 2-alkyl-5-hydroxy acyl chain that forms an amide bond with Leu.

A more complex interplay of PKS and NRPS enzymes can be envisaged in the biogenesis of the nostopeptolides from *Nostoc* (Golakoti *et al.*, 2000). Nostopeptolides A1 (Fig. 25) and A2 contain nine amino acid residues, including the nonproteinogenic amino acid 4-methylproline (mPro). Biogenesis starts with acylation (C_4 unit) of the starter Ile (A1) or Val (A2) and an additional acetate unit is inserted between two lecuine residues later in the extension process (Hoffmann *et al.*, 2003). The mPro is derived from a leucine residue, as in the nostocyclopeptolides (Section IV.D.1), also from *Nostoc* (Luesch *et al.*, 2003). Cyclization of the C-terminal proline with the hydroxyl group of serine is the final biosynthetic step (Hoffmann *et al.*, 2003).

Hassallidin A and B are antifungal glycosylated lipopeptides from *Hassallia* sp. Both variants contain a C_{14} acyl side chain linked to the macrocycle via Thr and a glycosylated threonine residue containing a mannose sugar; however the two variants differ in the composition of the side chain. Hassallidin A contains α,β-dihydroxytetradecanoic acid, whereas in hassallidin B (Fig. 25), the β-hydroxy group is glycosylated with a rhamnose moiety (Neuhof *et al.*, 2005, 2006).

3. *Cyclic Hybrid Depsipeptides Containing* β-*Amino Acids*

The cryptophycin/arenastatin groups of cyclic depsipeptides isolated from terrestrial *Nostoc* spp. are potent antitumor and antifungal agents (Fig. 26) (Chaganty *et al.*, 2004; Golakoti *et al.*, 1995). Because of their activity and mechanism of action by inhibition of microtubule polymerization (Kerksiek *et al.*, 1995), there has been a great deal of interest in the synthesis of analogues, including attempts to reengineer the biosynthetic pathway (Beck *et al.*, 2005). In general, all members of this group are composed of four units (A, B, C, and D). Unit A (7,8-epoxy-5-hydroxy- 6-methyl-8-phenyl-2-octenoic acid in cryptophycin 1) is a polyketide-derived moiety thought to be assembled from a Pla starter unit. The resulting acid is transferred to an NRPS-type protein where Tyr (unit B), methyl-β-alanine (unit C), and leucic acid (unit D)

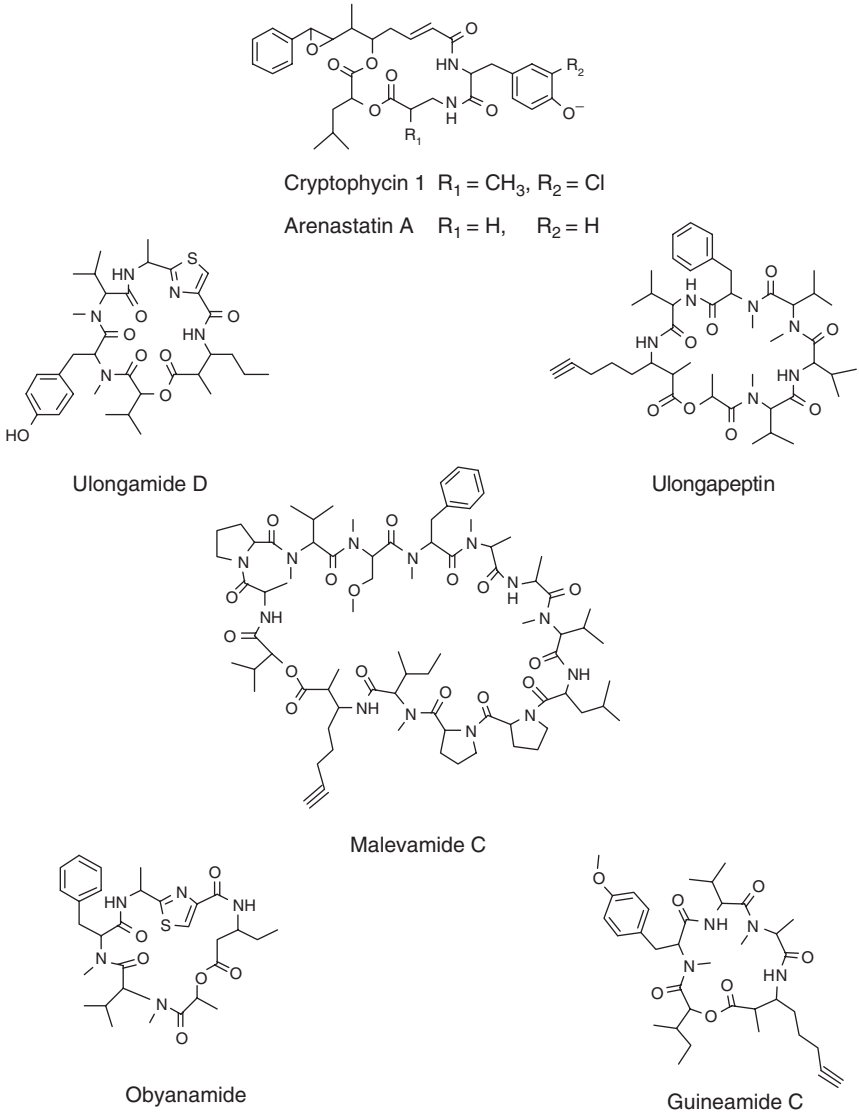


FIG. 26. Cyclic hybrid depsipeptides containing β-amino acids. All variants also contain an α-hydroxy acid.

are added (Beck *et al.*, 2005). Cryptophycin variants include derivatives containing nonchlorinated *O*-methylTyr or Phe with zero or two chlorine atoms at unit B, demethylated variants in unit C, and demethylated and hydrated derivatives of unit A (Schwartz *et al.*, 1990).

Structure-activity studies of cryptophycin 1 have shown that the intact macrolide ring structure, epoxide ring, the chloro- and *O*-methyl groups of tyrosine, and the methyl group at C2 in unit C are needed for *in vivo* activity. The synthetic cryptophycin analogue, cryptophycin 52, is currently in clinical trials as an experimental cancer treatment (Gerwick *et al.*, 2001; Golakoti *et al.*, 1995). Linear cryptophycins have also been isolated but determined to be artifacts of using methanol in the isolation process.

Ulongamides are cytotoxic thiazole-containing depsipeptides from *Lyngbya*. Ulongamides contain a variety of modified hydroxy and amino acids, for example ulongamide D contains 3-amino-2-methylhexanoic acid (Amha) and 2-hydroxyisovaleric acid (Hiv) (Fig. 26) (Luesch *et al.*, 2002b). Obyanamide is a cytotoxic depsipeptide from *Lyngbya* containing 3-aminopentanoic acid (Apa) as well as lactic acid (Fig. 26) (Williams *et al.*, 2002b).

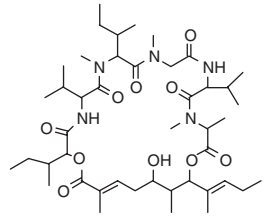
Malevamides B and C as well as ulongapeptin and guineamides all contain α-hydroxy acids in addition to β-amino acyl chains (Fig. 26). Malevamides B and C are cyclic depsipeptides from *Symploca* containing 3-amino-2-methyloct-7-ynoic acid (Amoa) or Amha as well as Hiv (Horgen *et al.*, 2000). Ulongapeptin, a cytotoxic depsipeptide from *Lyngbya*, contains 3-amino-2-methyl-oct-7-ynoic acid (AMO) and lactic acid (Williams *et al.*, 2003f). Guineamide, also from *Lyngbya*, contains 2-methyl-3-amino-oct-7-ynoic acid (Maoya) in addition to 2-hydroxy-3-methylpentanoic acid (Hmpa) (Tan *et al.*, 2003).

4. *Cyclic Dolastatins and Derivatives*

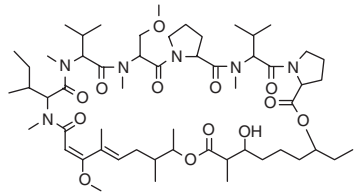
The aurilides (Han *et al.*, 2006) and lyngbyastatin 2, a dolastatin G analogue isolated from *Lyngbya majuscula* (Luesch *et al.*, 1999), share a common biogenetic signature in that the macrocyclic ring system contains two ester links (Fig. 27). In aurilide, a single presumptive polyketide chain participates in both ester linkages, whereas in lyngbyastatin 2, two polyketide-type hydroxy acid units, 3-methoxy-8-hydroxy-4,7-dimethylnon-2,4-dienoic acid (Nena), and 3,7-dihydroxy-2-methylnonanoic acid (Nana) provide the links.

Homodolastatin 16 is another cyclic hybrid depsipeptide dolastatin that has been isolated from the cyanobacterium *Lyngbya* (Fig. 27). This peptide contains a characteristic dolaphenvaline (Dpv) as well as dolamethylleucine (Dml) and Hiv (Davies-Coleman *et al.*, 2003).

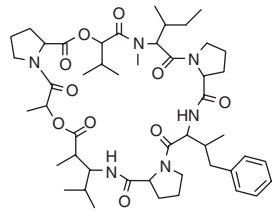
Dolastatin 11, from the sea hare *Dolabella*, as well as the cyanobacterial metabolites dolastatin 12, lyngbyastatin 1, lyngbyastatin 3, and majusculamide C share common structural features (Fig. 27). Majusculamide C is a cytotoxic and antifungal cyclic nonadepsipeptide isolated from



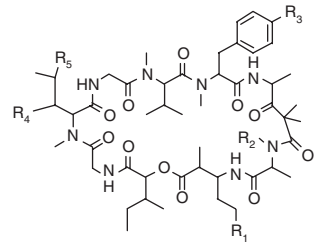
Aurilide B



Lyngbyastatin 2



Homodolastatin 16



Compound	R_1	R_2	R_3	R_4	R_5
Lyngbyastatin 3	CH_3	CH_3	OCH_3	H	CH_3
Lyngbyastatin 1	H	CH_3	OCH_3	H	CH_3
Dolastatin 12	H	CH_3	H	H	CH_3
Majusculamide C	H	H	OCH_3	CH_3	H
Dolastatin 11	H	H	OCH_3	H	CH_3

FIG. 27. Cyclic dolastatins and analogues isolated from cyanobacteria.

Lyngbya that shares the 4-amino-2,2-diemethyl-3-oxopentanoic acid (Ibu), 3-amino-2-methylpentanoic acid (MAP), 2-hydroxy-3-methylvaleric acid as well as the *N*,*O*-dimethylTyr found in dolastatin 11 (Carter *et al.*, 1984). Majusculamide C only differs from dolastatin 11 in that it contains *N*-methylIle rather than *N*-methylLeu. Dolastatin 12, which has been isolated from a *Lyngbya/Schizothrix* assemblage as well as from the sea hare *Dolabella*, contains *N*-methylPhe instead of *N*,*O*-diMeTyr, and the alanine residue is *N*-methylated. Lyngbyastatin 1 is an *N*-methylated variant of dolastatin 11, also at the alanine residue, and lyngbyastatin 3 is homologous to lyngbyastatin 1 except for the 3-amino-2-methylpentanoic acid unit is replaced by an Amha (Harrigan *et al.*, 1998b; Williams *et al.*, 2003b).

5. *Multicyclic Depsipeptides*

Multicyclic peptide structures are also found in cyanobacteria. The microviridins are tricyclic peptides isolated from *Microcystis*, *Oscillatoria*, and *Nostoc*. More than 10 microviridins have been reported, spanning a molecular weight range of 1600–1850 Da, making them the largest known cyanobacterial oligopeptides. The multicyclic structure of microviridins is formed by two ester links and one secondary amide link, and contains two side chains of variable length (Fig. 28). The major peptide ring contains seven amino acids with an ester bond between the side chain carboxyl group of aspartate and the hydroxyl group of threonine, and a peptide bond between the side chain amine group of lysine and the side chain carboxyl group of glutamate. Structural variations in microviridins are usually the result of substitutions in the longer side chain and position 5 (Leu in microviridin B, Tyr in microviridin E) in the ring. Variations also occur when one or both ester linkages are not intact, resulting in methyl ester variants that could possibly be an artifact similar to those seen for the cryptophycins (Ishitsuka *et al.*, 1990; Murakami *et al.*, 1997c; Okino *et al.*, 1995; Rohrlack *et al.*, 2004; Shin *et al.*, 1996b). All amino acids in microviridins are found in the L-configuration. It has been suggested that microviridins are synthesized ribosomally and the tricyclic structure is completed by posttranslational modifications, similar to microcin J25 produced by *E. coli* (Blond *et al.*, 1999; Welker and von Doehren, 2006).

V. Alkaloids

Cyanobacteria produce a great variety of alkaloids. Like the peptides, the alkaloids are often assembled from amino acids in what appears to be hybrid PKS/NRPS pathways. The most prolific sources of alkaloids are

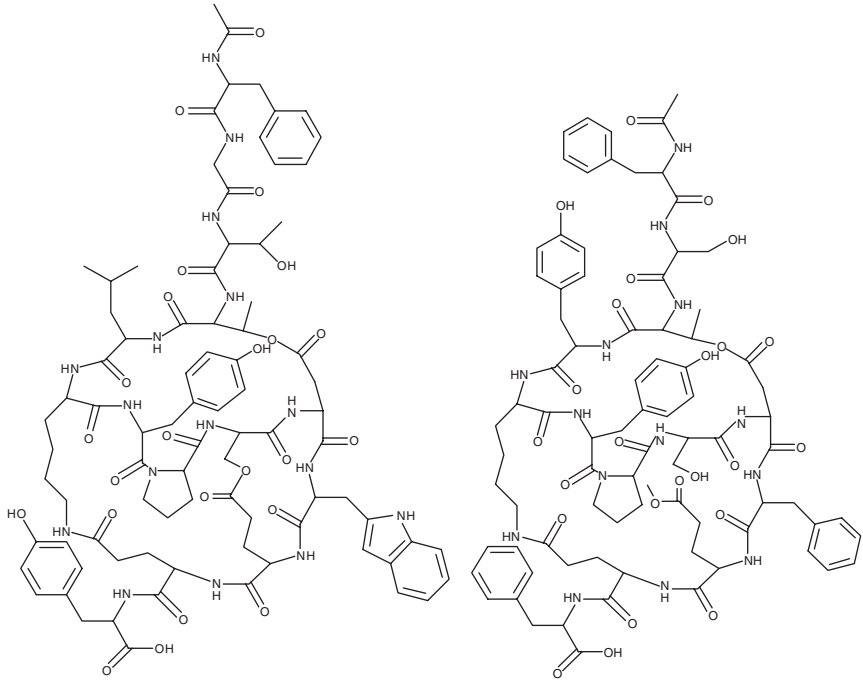


FIG. 28. Structures of microviridins B and E, multicyclic depsipeptides isolated from cyanobacteria.

marine isolates of *Lyngbya*. Some recurrent features among cyanobacterial alkaloids are the presence of thiazole rings, halogenation (particularly bromination of indoles and trichlorination of methyl groups), prenylated indoles, pyrrololactams, cyclic guanidiniums, and small bicyclic structures. As is the case in the polyketides, there are some cyanobacterial alkaloids that strongly resemble alkaloids from actinomycetes. A thorough review of the alkaloids reported from cyanobacteria that includes discussion on aspects of stereochemistry and structure determination was published and should be consulted for additional information (Gerwick *et al.*, 2001).

A. LINEAR ALKALOIDS

A small number of linear alkaloids, derived primarily from PKS-like reactions and usually containing a single amide link, have been reported from cyanobacteria. An unusual family of polychlorinated acetamides

(Fig. 29A) was reported from *Microcoleus* containing alkyne groups and chlorinated aliphatic carbons (Orsini *et al.*, 2001). A plausible mechanism of chlorination would involve chlorination of a carbon atom adjacent to a carbonyl. The biosynthetic pathway for the actinomycete metabolites neocarzilins produces an α-trichloromethyl ketone possibly using a flavin-dependent halogenase (Otsuka *et al.*, 2004). Alternatively, another possible chlorination mechanism is discussed in Section V.B.3. Some of the polychlorinated acetamides have an odd number of carbons in their chains, suggesting decarboxylation of a biosynthetic intermediate.

Pitiamide A, a linear lipopeptide that has a chloroalkene terminus (Fig. 29B), was isolated from an assemblage of *Lyngbya* and *Microcoleus* growing on an intact coral (Nagle *et al.*, 1997). This compound exhibited antifeedant properties against a common reef-feeding fish. Pitiamide appears to derive from a mixed PKS/NRPS pathway.

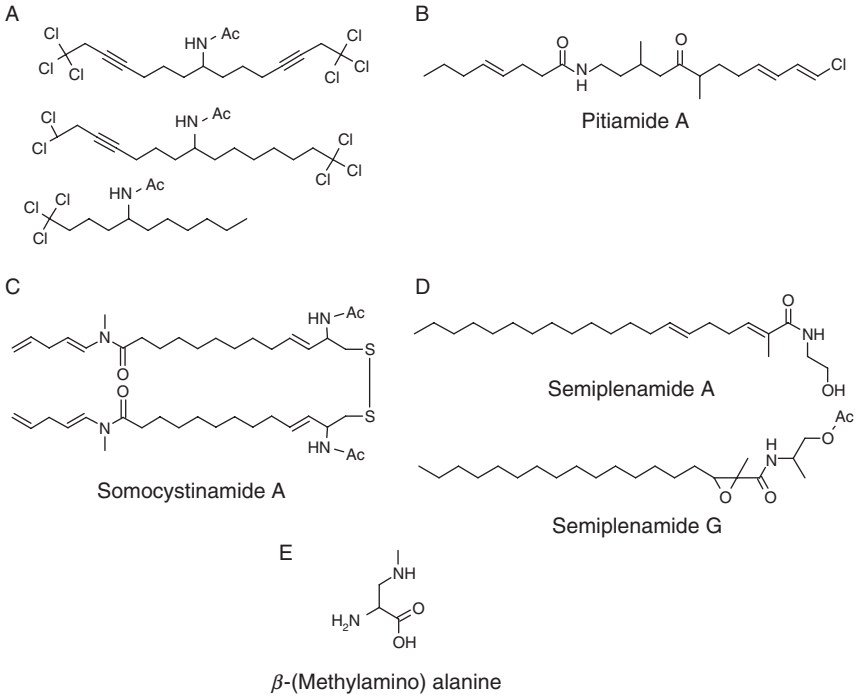


FIG. 29. Examples of the linear alkaloids reported from cyanobacteria. (A) Chlorinated alkylamines from *Microcoleus*. (B) Pitiamide A. (C) Somocystinamide A. (D) Two examples of the semiplenamides. (E) β-(methylamino) alanine from various cyanobacteria. This compound has been associated with neurodegeneration among inhabitants of Guam.

Nogle and Gerwick (2002a) reported somocystinamide A (Fig. 29C) from a *Lyngbya*/*Schizothrix* assemblage that appears to result from a hybrid PKS/NRPS pathway. Somocystinamide A is a symmetric disulfide-linked dimer. The longest chain apparently results from a cysteine starter unit condensed with acetate, and then elongation as in normal PKS synthesis. The nascent acyl chain undergoes a second hybrid coupling with a second unidentified amino acid to form the amide bond. This structure highlights one aspect of the flexibility of hybrid PKS/NRPS systems in that there are no simple couplings of one amino acid to another in this molecule as would typically be seen in NRPS or ribosomal pathways.

The semiplenamides from *Lyngbya* (Han *et al.*, 2003) are a series of amide derivatives of 2-methyl fatty acids with either a double bond or epoxide between carbons 2 and 3 (Fig. 29D). An additional double bond is sometimes present at position 6. Chain lengths of 16, 18, and 20 have been observed, and the amine groups are variably acetylated derivatives of ethanolamine or alaninol. They have been reported to have toxic and anandamide membrane transporter inhibitory activities.

β-(Methylamino) alanine (BMAA; Fig. 29E) is a compound which, despite its uncomplicated structure, may have far reaching implications for public health. A study found that this neurotoxin is produced by cyanobacteria and ''biomagnified'' through the food chain of a traditional Guam diet by way of *Cycad* seeds and flying foxes (Cox *et al.*, 2003). The neurotoxin was further found to be present in high levels in sufferers of amyotrophic lateral sclerosis/Parkinson's dementia complex, which has a significantly higher incidence among people living in Guam (Murch *et al.*, 2004a). Moreover, production of BMAA appears to be widespread across all groups of cyanobacteria (Cox *et al.*, 2005). These circumstances warrant further study into levels of BMAA near cyanobacterial blooms and, more importantly, in human food sources exposed to such blooms. While studies with mice have called into question the level of neurotoxicity achieved by dietary BMAA (Cruz-Aguado *et al.*, 2006), studies with spinal cord cell cultures suggest potent toxicity perhaps via AMPA/kainate channel activation (Rao *et al.*, 2006). Although BMAA is highly polar and hence not expected to accumulate over time, a study has shown that BMAA does accumulate such that it can be released by acid, suggesting incorporation into proteins and slow release by their degradation (Murch *et al.*, 2004b). Given that BMAA contains two amine groups close to one another that can condense with other amino acids, more studies are needed to explore the effect that BMAA incorporation into proteins by ribosomal synthesis has on the structure and folding stability of those proteins.

B. Ring-Containing Alkaloids

1. *Amides of Lyngbic Acid*

The malyngamides (Fig. 30) are a large class of compounds from *Lyngbya* apparently derived from hybrid PKS/NRPS pathways that utilize lyngbic acid or closely related fatty acids as starter units. Two major classes of malyngamides are discernible from their structures: those that contain cyclohexanone in their amide portion, and those that contain pyrrololactam derivatives. The first class has been postulated to derive from extension of the lyngbic acid group with glycine or β-alanine followed by extension with malonate groups and a cyclizing Claisen condensation (Fig. 30A) (McPhail and Gerwick, 2003; Nogle and Gerwick, 2003). Many members of this class possess pendant methyl groups attached to putative carbonyl carbons, often present as a chloroalkene group. Chloroalkene substitution is often seen in the latter class of malyngamides, which appear to derive from a similar pathway to the first family, except extension by two malonates is followed instead by a second NRPS addition, perhaps using proline or glycine or serine to form the heterocycle. A few of the dozens of malyngamides characterized so far are shown in Fig. 30B (Ainslie *et al.*, 1985; Cardellina *et al.*, 1978, 1979b; Christophersen, 1985; Gerwick *et al.*, 1987; Kan *et al.*, 2000; McPhail and Gerwick, 2003; Milligan *et al.*, 2000a; Mynderse and Moore, 1978a; Nogle and Gerwick, 2003; Orjala *et al.*, 1995a; Praud *et al.*, 1993; Todd and Gerwick, 1995b; Wan and Erickson, 1999; Wu *et al.*, 1997). Also shown is one of the hermitamides isolated from *Lyngbya* (Tan *et al.*, 2000), which are likewise derived from condensation of lyngbic acid with an amino acid derivative. Compounds similar to the malyngamides have also been isolated from mollusks (Appleton *et al.*, 2002; Gallimore and Scheuer, 2000) and red algae (Kan *et al.*, 1998).

2. *Lactone-Containing Alkaloids*

Laingolide (Klein *et al.*, 1996), laingolide A, and madangolide (Fig. 31A) (Klein *et al.*, 1999b) are cyclic lactones from *Lyngbya* that appear to be derived by insertion of a single glycine moiety in a polyketide chain, a feature that has been observed in dinoflagellate metabolites (Macpherson *et al.*, 2003). The laingolides contain a 4-aminobut-3-enoic acid moiety in contrast to the 4-aminobut-2-enoic acid moiety which is more frequently encountered in cyanobacteria (Klein *et al.*, 1996). All of the compounds contain an unusual *tert*-butyl side chain which may derive from the starter unit of the PKS chain, as postulated for antillatoxin and apratoxin (Fig. 25). The compounds all appear to undergo methylation at carbons

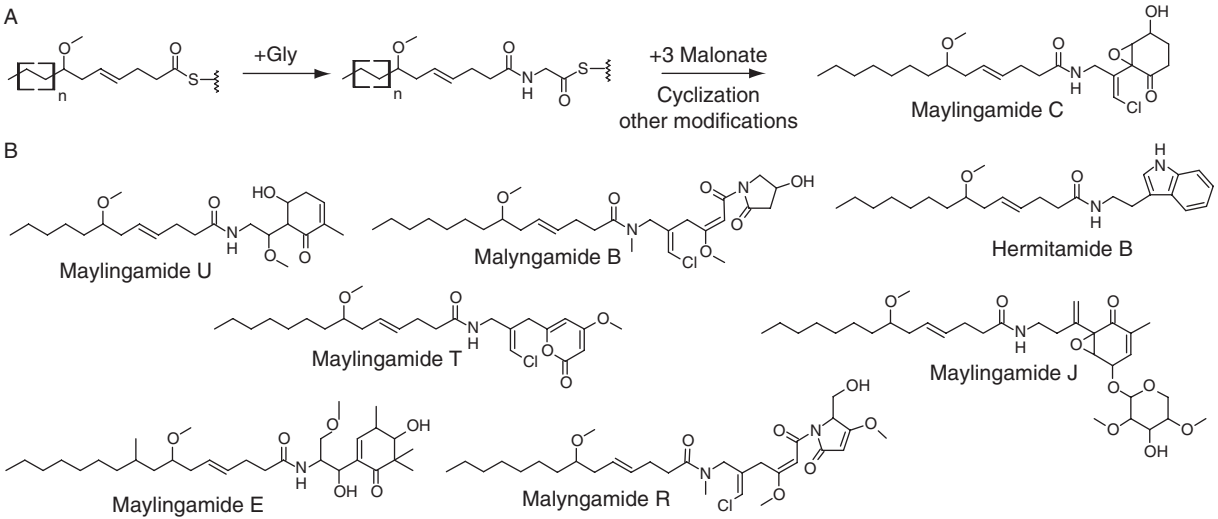


FIG. 30. Malyngamides. (A) Hypothetical biogenesis for cyclohexanone-containing malyngamides. (B) Representative structures of the malyngamides and hermitamides. References for the compounds are as follows: malyngamide B (Cardellina *et al.*, 1978), malyngamide C (Ainslie *et al.*, 1985), malyngamide E (Mynderse and Moore, 1978a), malyngamide J (Wu *et al.*, 1997), malyngamide R (Milligan *et al.*, 2000a), malyngamide T (Nogle and Gerwick, 2003), malyngamide U (McPhail and Gerwick, 2003), and hermitamide B (Tan *et al.*, 2000).

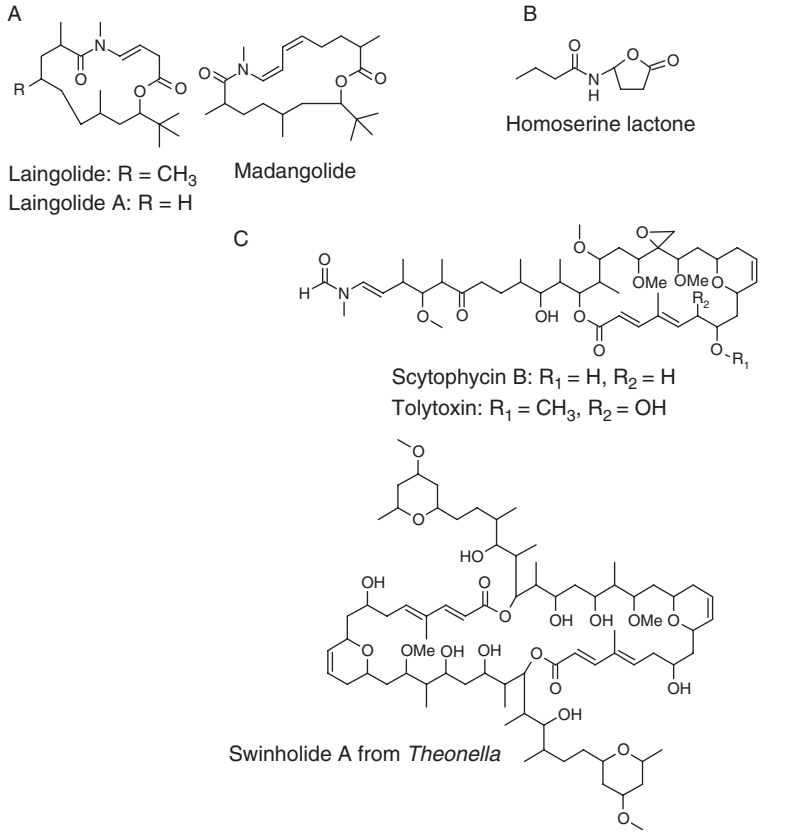


FIG. 31. Several lactone alkaloids from cyanobacteria and related compounds. (A) Langolides and madangolide. (B) An acylhomoserine lactone from cyanobacteria. (C) Scytophycin B and tolytoxin. Also shown is swinholide A, a related macrolide found in cyanobacteria and sponges.

derived from both C1 and C2 of acetate, another phenomenon observed in dinoflagellates (Macpherson *et al.*, 2003; Sato *et al.*, 2000).

A homoserine lactone (HSL) analogue (Fig. 31B) was reported from *Lyngbya* (Marner and Moore, 1978), and is similar to HSLs that act as quorum-sensing molecules in Gram-negative bacteria (Whitehead *et al.*, 2001). It is not known if these types of molecules have a signaling role in cyanobacteria.

The scytophycins and the closely related tolytoxin (Fig. 31C) are a family of eneamide-containing macrolides from *Scytonema* (Carmeli *et al.*, 1990b; Ishibashi *et al.*, 1986; Moore *et al.*, 1986b) and from

Cylindrospermum (Jung *et al.*, 1991). Feeding studies with stable isotopically labeled precursors showed tolytoxin to result from a mixed PKS/NRPS pathway in which the starter unit is glycine and that the various methyl branch carbons are derived from the tetrahydrofolate C_1 pool (Carmeli *et al.*, 1993a). The various members of the family differ from one another primarily in oxidation state and methylation state of the alcohol, ether, and ketone functionalities. The scytophycins strongly resemble the monomer skeleton of the swinholides which were originally reported from the lithistid sponge *Theonella swinhoei* (Carmely and Kashman, 1985; Carmely *et al.*, 1986; Kitagawa *et al.*, 1990). Lithistid sponges contain stable communities of cyanobacteria and other microbes. However, chemical studies on separated cell fractions from *Theonella swinhoei* found swinholide A to be located only in ''the mixed population of unicellular heterotrophic bacteria'' with no compounds present in the cyanobacterial fraction (Bewley *et al.*, 1996), though a report describes the isolation of swinholide A from a *Symploca* cyanobacterium (Andrianasolo *et al.*, 2005).

3. *Alkaloids Containing Small Heterocyclic Rings*

Kalkitoxin (Fig. 32A) is a neurotoxic, ichthyotoxic compound of mixed PKS/NRPS origin that was isolated from *Lyngbya* (Berman *et al.*, 1999; Wu *et al.*, 2000). Kalkitoxin contains a thiazoline ring whose biogenesis is described in Section II.B.1. Other notable features in kalkitoxin include a terminal monosubstituted olefin group, probably derived via decarboxylation of the terminal acetate unit, and adjacent methylated carbons in the central polyketide portion of the molecule. Because of the different reactivities of the keto and methyl carbons in polyketide intermediates, methylation reactions at the two sites necessarily proceed via different mechanisms from one another. By far, SAM-mediated methylation of methyl carbons of polyketide intermediates is the most common.

The curacins from *Lyngbya* (Fig. 32B) are a family of thiazoline-containing compounds resulting from mixed PKS/NRPS biosynthesis (Gerwick *et al.*, 1994; Márquez *et al.*, 1998; Nagle *et al.*, 1995; Yoo and Gerwick, 1995). The curacins differ from one another based on double bond geometry or methyl branching of the side chain. The curacins inhibit microtubule polymerization by binding to the colchicine site on tubulin (Blokhin *et al.*, 1995). The gene cluster required for curacin A biosynthesis was cloned (Chang *et al.*, 2004) and found to contain multiple PKS genes, most of which are single modules, and a gene with mixed PKS/NRPS domains that appears to be involved in thiazolidine formation from cysteine. Isotopic feeding experiments indicated that

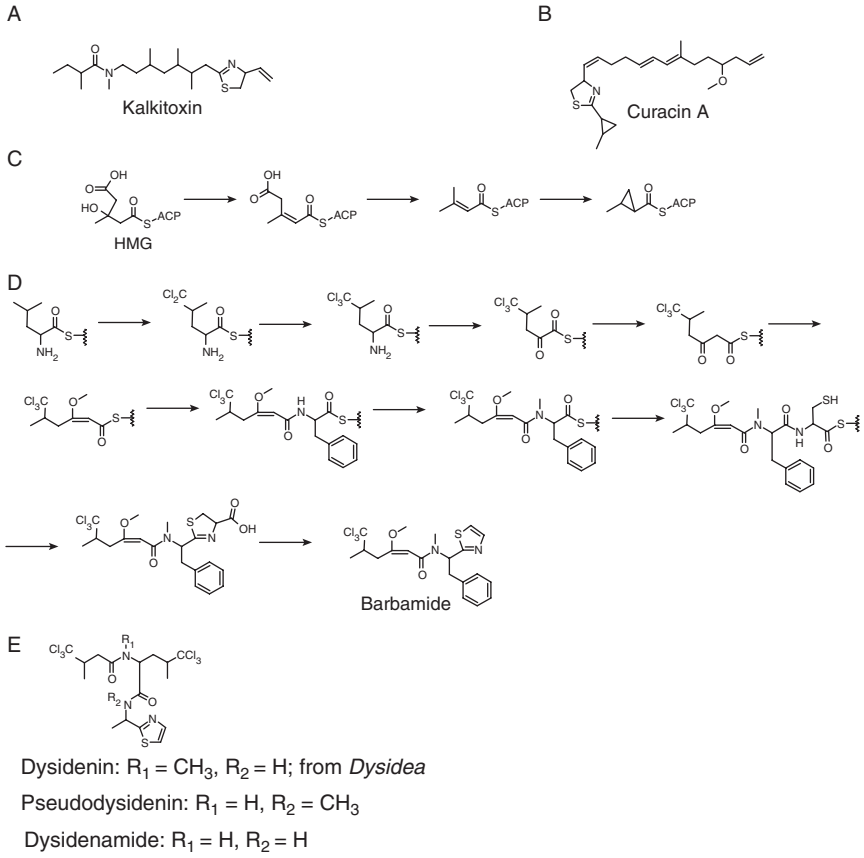


FIG. 32. Thiazole-containing cyanobacterial metabolites. (A) Kalkitoxin. (B) Curacin A. (C) Proposed biogenetic route to the cyclopropyl ring in curacin A. (D) Sequence of intermediates for the biosynthesis of barbamide. (E) Dysidenin from the sponge *Dysidea* and related metabolites from *Lyngbya*.

biosynthesis starts at the cyclopropyl end of the molecule and proceeds toward the long chain. An acetate group is condensed directly with the carboxyl group of the amino acid residue. Formation of the cyclopropyl ring is believed to proceed via a 3-hydroxy-3-methylglutaryl (HMG) intermediate by dehydration followed by decarboxylation and cyclization (Fig. 32C) (Gu *et al.*, 2006). Decarboxylation also occurs at the other end of the molecule to yield the terminal double bond.

Barbamide (Fig. 32D) from *Lyngbya* (Orjala and Gerwick, 1996) contains a trichloromethyl group in addition to a fully oxidized thiazole ring.

Stable isotope feeding studies revealed that the chlorinated carbon in barbamide derives from the pro-R methyl group of leucine, and also that halogenation occurs directly on the methyl group *without* activation via olefin formation (Sitachitta *et al.*, 1998, 2000a). These studies further showed the carbons of barbamide to originate from leucine, acetate, phenylalanine, cysteine, and the C_1 methyl pool. The biosynthetic pathway producing barbamide was later cloned and shown to encode a mixed PKS/NRPS system with tailoring enzymes (Chang *et al.*, 2002). Subsequent studies established that trichlorination of the methyl group proceeds through tandem reactions performed by two nonheme Fe^{II} halogenases which operate by a free radical mechanism (Galonić *et al.*, 2006). One enzyme, BarB2, adds the first two chlorine atoms and the second enzyme, BarB1, is required to add the third. These findings were supported by another study utilizing stable isotopic feeding experiments that also suggested the addition of the first two chlorine atoms to be a separate event from the addition of the third (Flatt *et al.*, 2006). The trichloroleucine residue serves as the starter unit for the PKS/NRPS pathway. Unusually, the subsequent condensation with malonate involves the α-carbon of the leucine derivative rather than with the carboxylate carbon, most likely on an α-keto intermediate formed by the putative deaminase BarJ (Chang *et al.*, 2002). The enolate form of the diketide is trapped by *O*-methylation. The chain is subsequently elongated with phenylalanine and finally cysteine which, in addition to cyclization to a thiazoline, appears to undergo oxidative decarboxylation to give the final thiazole. A scheme illustrating the probable sequence of intermediates (Chang *et al.*, 2002) is shown in Fig. 32D.

Several small linear peptides with trichloromethyl groups have been reported from *Lyngbya* as well. The largest family of such peptides are the dysidenins (Fig. 32E) (Jiménez and Scheuer, 2001), so named because the first compound in this family was isolated from a *Dysidea* sponge (Kazlauskas *et al.*, 1977). The dysidenins isolated from sponges are structurally indistinct from those isolated from cyanobacteria, suggesting that the dysidenins are produced by cyanobacteria associated with the sponges. There was evidence of chlorinated metabolites of *Dysidea* being localized in cyanobacterial symbionts of the sponge before the first reported isolation of a dysidenin from a cyanobacterium (Flowers *et al.*, 1998; Unson and Faulkner, 1993). The biosynthesis of the dysidenins probably resembles the barbamide pathway. Again, there is a trichloroleucine starter unit that undergoes condensation to its α-carbon, this time to a second trichloroleucine unit. This is followed by condensation with alanine, then cysteine, then cyclization,

and oxidative decarboxylation to form a thiazole. Methylation also occurs for amide nitrogens in some cases.

Ypaoamide (Fig. 33A) (Nagle *et al.*, 1996), which was isolated from a mixed assemblage of *Lyngbya* and *Schizothrix*, is a compound that shares some common structural features with barbamide. Both compounds contain β-methoxy, α,β-unsaturated amide groups and both compounds result from mixed PKS/NRPS pathways. A distinctive feature of ypaoamide is an acyl chain with a terminal *tert*-butyl group, an unusual but recurrent motif in cyanobacterial products. The heterocycle in ypaoamide appears to result from extension of the tyrosine residue with an acetate unit followed by cyclization. A similar compound, palau'imide (Fig. 33A), was also reported from *Lyngbya* (Luesch *et al.*, 2002d). In both cases, cleavage of the product from the PKS/NRPS appears to occur by way of attack by the nitrogen of an aromatic amino acid on the terminal acetate carbonyl, producing a pyrrolimide.

The pukeleimides from *Lyngbya* (Fig. 33B) also contain a pyrrolimide substructure (Cardellina and Moore, 1979; Simmons *et al.*, 1979). In the case of the pukeleimides, the penultimate residue appears to be glycine rather than an aromatic residue. The biosynthetic origin of the rest of the molecule appears to have no precedent.

The fischerellins (Fig. 33C), isolated from *Fischerella*, are enediyne metabolites that show activity against photosynthetic microbes, including cyanobacteria (Gross *et al.*, 1991; Hagmann and Jüttner, 1996). The configuration of the fischerellin enediyne moiety (ene/yne/yne) is topologically distinct from that seen in the more famous anticancer enediynes (yne/ene/yne) from actinomycetes. An interesting difference between fischerellin A and B is the presence of methyl branching on the side chain in the former, and absence of methylation but extension of the side chain by one carbon in the latter. No biosynthetic studies have been undertaken for the fischerellins.

The tumonoic acids (some of which are shown in Fig. 33D) are acyclic depsipeptides isolated from a *Lyngbya*/*Schizothrix* assemblage (Harrigan *et al.*, 1999). The most distinctive feature of these compounds is the presence of esters of various α-hydroxy acids, such as Hiv, isoleucic acid, and lactic acid, which are often found in cyanobacterial peptides such as mirabimide A (Section. IV.C.1) and the tasiamides (Section IV.C.2), and are likely derived from the corresponding amino acid. The polyketide chain is a unique 2,4-dimethyl-3-hydroxydodec-4-enoic acid (Doda), and the metabolite itself is likely derived from a mixed PKS/NRPS pathway that incorporates proline. Another family of peptides that includes ester bonds to amino acid homologues are the lyngbyabellins from *Lyngbya* (Section IV.E.2) and their ring-opened

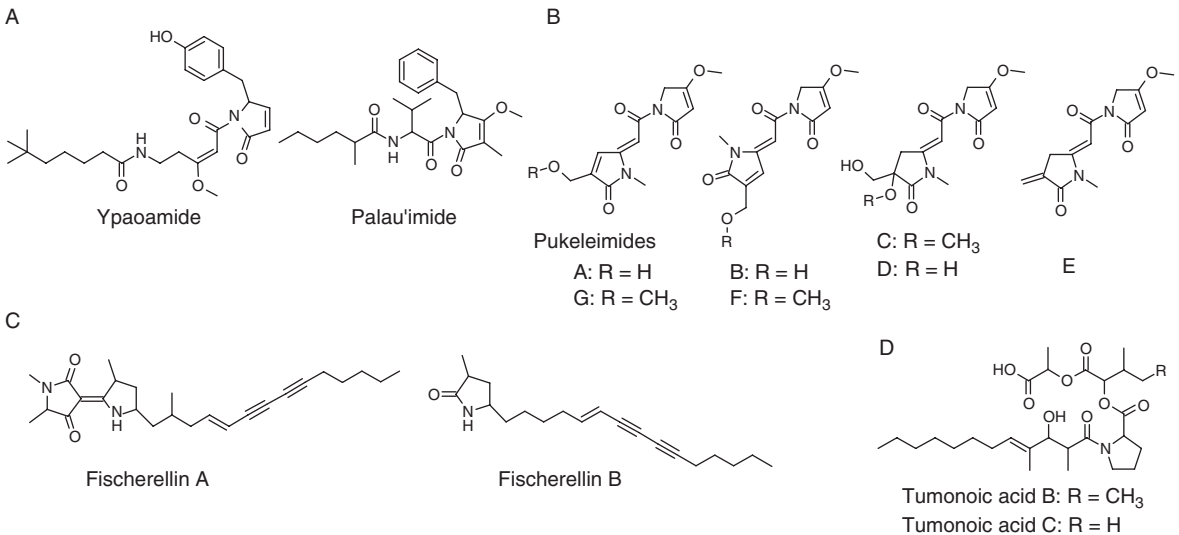


FIG. 33. Pyrrole-containing cyanobacterial metabolites. (A) Ypaoamide and palau'imide. (B) The pukeleimides. (C) The fischerellins. (D) Tumonoic acids B and C.

analogues (Han *et al.*, 2005b; Luesch *et al.*, 2000b,c,d; Marquez *et al.*, 2002; Milligan *et al.*, 2000b; Williams *et al.*, 2003a).

4. *Pigments and Photoprotectants*

As photosynthetic organisms, cyanobacteria contain chlorophyll and other porphyrin derivatives. *Anacystis* contains a compound that appears to be a Baeyer–Villiger oxidative ring expansion product of chlorophyll *a* (Fig. 34A) (Wu and Rebeiz, 1988). The tolyporphins are another class of heme-containing porphyrins (Fig. 34B). The tolyporphins were all isolated from *Tolypothrix* (Prinsep *et al.*, 1992a, 1995, 1998; Minehan *et al.*, 1999) and have multidrug resistance-reversing (Smith *et al.*, 1994) and tumor-photosensitizing activities (Morliere *et al.*, 1998). Several of the tolyporphins contain one or two C-glycosidic groups attached to the heme ring. In general, C-glycoside biosynthesis is proposed to occur through attack by a carbon nucleophile on a nucleoside diphosphate-activated sugar (Bililign *et al.*, 2005), and such a mechanism would appear reasonable in the case of the tolyporphins. Indeed, C-glycosylation is mechanistically similar to methylation by SAM, so similar sites are likely to be reactive. Other members of the tolyporphins have oxygen atoms attached to one or both of the glycosylation sites. Variation within the family consists mainly of substitution at these sites and acetylation of the various alcohol groups.

Phycocyanobilin (Fig. 34C) is a protein cofactor derived from oxidative ring opening of heme that forms part of the light harvesting machinery of cyanobacteria. The distinctive blue color of phycocyanobilin accounts for the original name of ''blue–green algae'' given to the cyanobacteria. Unlike the cyclic hemes, the linear bilins do not bind metal ions tightly.

Pterins, small heteroaromatic compounds distributed widely in nature, participate in several enzymatic activities and attracted much early interest as pigments in butterfly wings. Pterin glycosides have been found in cyanobacteria (Fig. 35A), such as the glucoside of biopterin which was isolated from *Spirulina* and found to protect chlorophyll *a*, phycocyanin, and carotenoids from photobleaching by UV irradiation (Noguchi *et al.*, 1999). A diglycoside cyanopterin isolated from *Synechocystis* was present at comparable levels to chlorophyll *a* (Lee *et al.*, 1999a). Experiments probing the intracellular oxidative state of cyanopterin suggested that most of it is present as the reduced, tetrahydro state. While the physiological function of the pterin glycosides is not known, studies suggest that glycosylation greatly increases the achievable intracellular concentrations of the biopterins (Choi *et al.*, 2001; Hwang *et al.*, 2002). A photoprotective role for the biopterins is suggested based on observations of stabilization of cellular

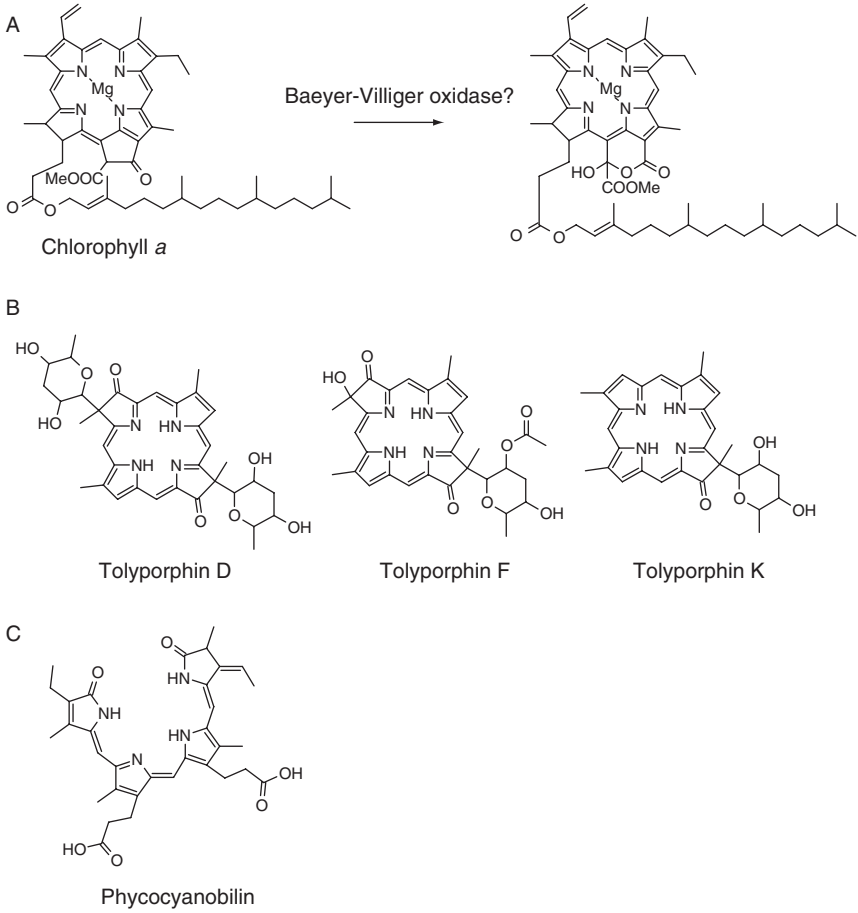


FIG. 34. Porphyrin-containing cyanobacterial metabolites. (A) A porphyrin from *Anacystis* and its possible derivation from chlorophyll *a*. (B) Examples of the tolyporphins. (C) Phycocyanobilin.

pigments against irradiation (Noguchi *et al.*, 1999; Saito *et al.*, 2003) and increases in biopterin glycoside production in response to UV-A irradiation (Wachi *et al.*, 1995). Some work has been done on identifying the genes responsible for the production of the pterin glycosides in *Synechocystis* (Chung *et al.*, 2000; Choi *et al.*, 2001; Hwang *et al.*, 2002; Lee *et al.*, 1999c).

Mycosporine-like amino acids (MAAs) (Fig. 35B) are another class of photoprotectants commonly found in cyanobacteria. The MAAs are

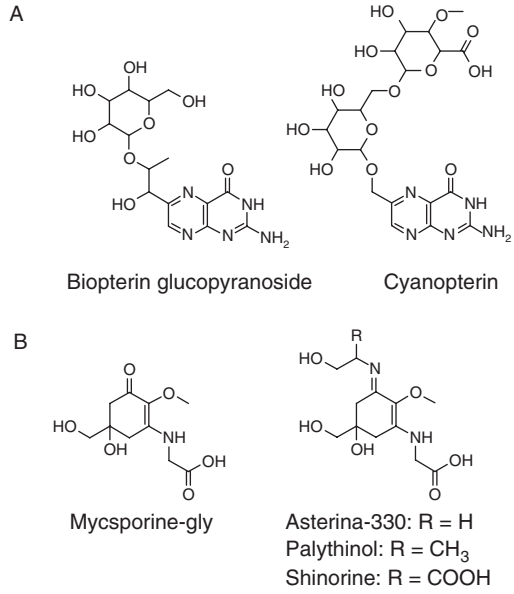


FIG. 35. Other pigments from cyanobacteria. (A) Pteridine pigments. (B) Mycosporine-like amino acid pigments.

small cyclohexenone derivatives or cyclohexenimine derivatives that are transparent to visible light but strongly absorb UV light in the 300–360 nm range (Shick and Dunlap, 2002). The MAAs are synthesized by the attachment of amino acids to the cyclohexenone ring, which is derived from the shikimate pathway (Shick and Dunlap, 2002). MAAs reported from cyanobacteria include mycosporine-gly in *Nostoc* (Ehling-Schulz *et al.*, 1997) and *Gloeocapsa* (Sommaruga and Garcia-Pichel, 1999); asterina-330 from *Gloeocapsa* (Sommaruga and Garcia-Pichel, 1999); palythinol from *Gloeocapsa* (Sommaruga and Garcia-Pichel, 1999); and shinorine from *Anabaena* (Sinha *et al.*, 1999), *Gloeocapsa* (Sommaruga and Garcia-Pichel, 1999), *Lyngbya* (Karsten *et al.*, 1998), and *Microcoleus* (Karsten and Garcia-Pichel, 1996). An online database of various photoprotectant compounds in cyanobacteria, phytoplankton, and microalgae provides useful information for the MAAs and scytonemin (Section V.B.5; Gröniger *et al.*, 2000).

5. *Indole Alkaloids*

Some small indole derivatives have been reported from *Lyngbya* (Fig. 36A). The compound *N*,7-dimethylindole-3-carboxaldehyde (Todd and Gerwick, 1995a) was the first reported aldehyde from a

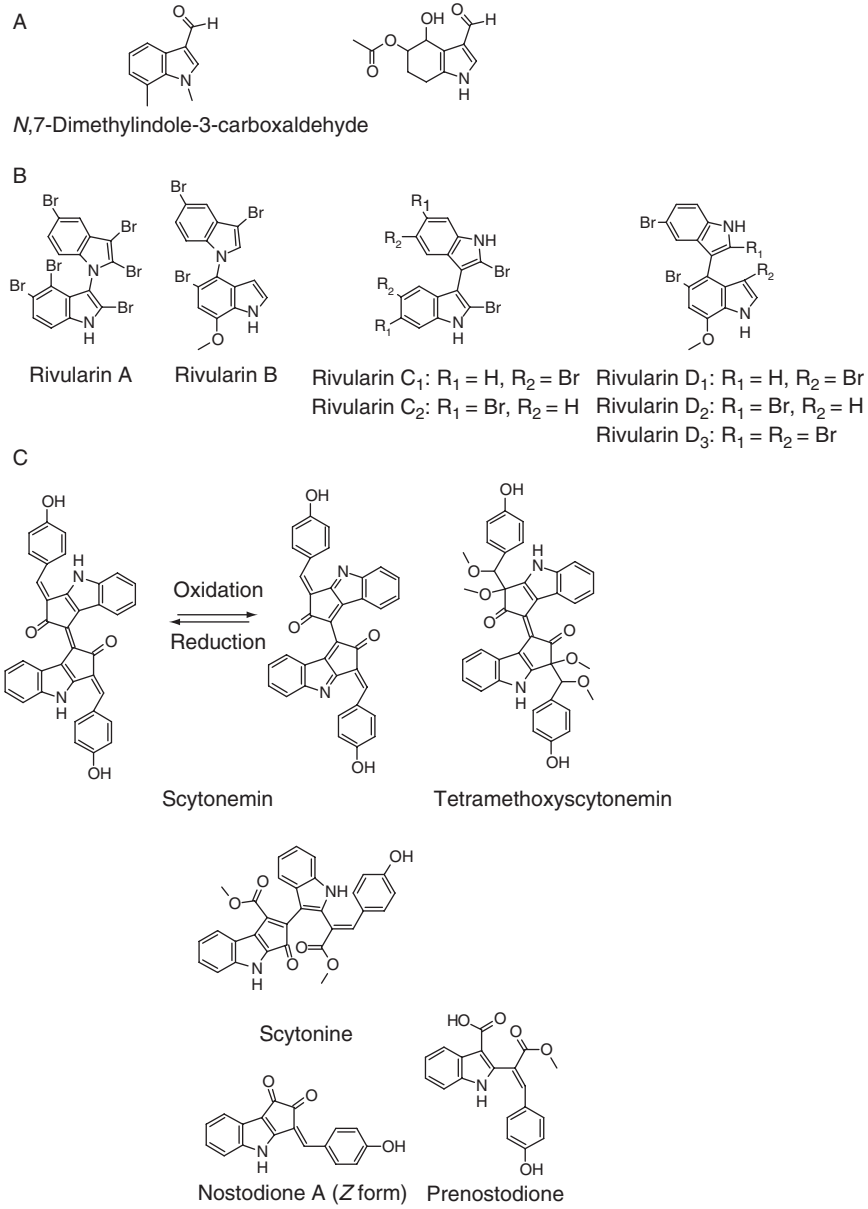


FIG. 36. Several indole alkaloids from cyanobacteria. (A) Simple indole derivatives. (B) Examples of the rivularins. (C) Scytonemin and related alkaloids.

cyanobacterium. The sites of methylation are also unusual among algae. A related aldehyde with a reduced aryl ring was later isolated from the same genus (Nogle and Gerwick, 2003).

The rivularins, a series of polybrominated indole dimers (Fig. 36B), have been reported from Australian samples of *Rivularia* (Hodder and Capon, 1991b; Norton and Wells, 1982). Despite their planar structure, several derivatives exhibit chirality due to hindered rotation around the bicyclic bond. The compounds have been named according to the sites of dimerization (Maehr and Smallheer, 1984): A = 1,3′; B = 1,4′; C = 3,3′; and D = 3,4′. In most (but not all) cases, both sites of dimerization are directly adjacent to brominated carbons. All rivularins that dimerize at 4′ are methoxylated at 7′.

Scytonemin (Fig. 36C), which is found in the extracellular sheath of more than 30 species of cyanobacteria, is believed to serve a photoprotective function. Unfortunately there is a cyclic peptide named scytonemin A that has no structural or functional relation to scytonemin. Although scytonemin was first noted as a pigment in 1877 (Nägeli and Schwenderer, 1887), the structure was not determined until 1993 (Proteau *et al.*, 1993) due to its structural complexity. Scytonemin occurs both as a reduced form, which is red, and an oxidized form, which is green. Reduction of the oxidized form occurs with ascorbic acid; oxidation of the reduced form occurs in air on silica gel (Proteau *et al.*, 1993). Many studies have demonstrated the ability of scytonemin to protect cyanobacteria from UV-A radiation (Brenowitz and Castenholz, 1997; Dillon *et al.*, 2002; Garcia-Pichel and Castenholz, 1991; Garcia-Pichel *et al.*, 1992; Ivanov *et al.*, 2000; Quesada *et al.*, 1999). The wide distribution of this pigment within cyanobacteria and the ability of the compound to protect DNA and photosynthetic machinery against UV-C radiation, which is largely filtered out in our oxygen- and ozone-rich atmosphere, have led researchers to suggest that scytonemin may have protected early cyanobacteria that existed on the planet before there was an aerobic atmosphere (Dillon and Castenholz, 1999). Methoxylated compounds and a cleavage/isomerization product (scytonine) related to scytonemin (Fig. 36C) have been reported from *Scytonema* (Bultel-Ponce *et al.*, 2004). Other compounds, namely nostodione (Kobayashi *et al.*, 1994) and prenostodione (Ploutno and Carmeli, 2001) both from *Nostoc*, clearly have structures related to scytonemin (Fig. 36C). The compounds may originate from scytonemin by sequential oxidative bond cleavages. However, the presence of *E* and *Z* double bond isomers in nostodione and the presence of only an *E* isomer in prenostodione led to a hypothetical scheme in which prenostodione serves as a biosynthetic precursor to nostodione (Ploutno and Carmeli, 2001). Little is

known about the biosynthesis of scytonemin other than the presence of tryptophan- and tyrosine-like structures in the product.

Several indole-containing carbazoles have been reported from cyanobacteria, the largest family of which are the antifungal tjipanazoles from *Tolypothrix* (Bonjouklian *et al.*, 1991). The tjipanazoles are structurally very similar to actinomycete products rebeccamycin (Nettleton *et al.*, 1985) and staurosporine (Omura *et al.*, 1977). The biosynthetic pathways for the production of rebeccamycin (Sánchez *et al.*, 2002; Onaka *et al.*, 2003) and staurosporine (Onaka *et al.*, 2005) have both been cloned and heterologously expressed, and various aspects of the biosynthetic pathway have emerged (Howard-Jones and Walsh, 2005; Nishizawa *et al.*, 2005, 2006; Onaka *et al.*, 2003, 2005; Sánchez *et al.*, 2002; Yeh *et al.*, 2005, 2006), a summary of which is shown in Fig. 37A.

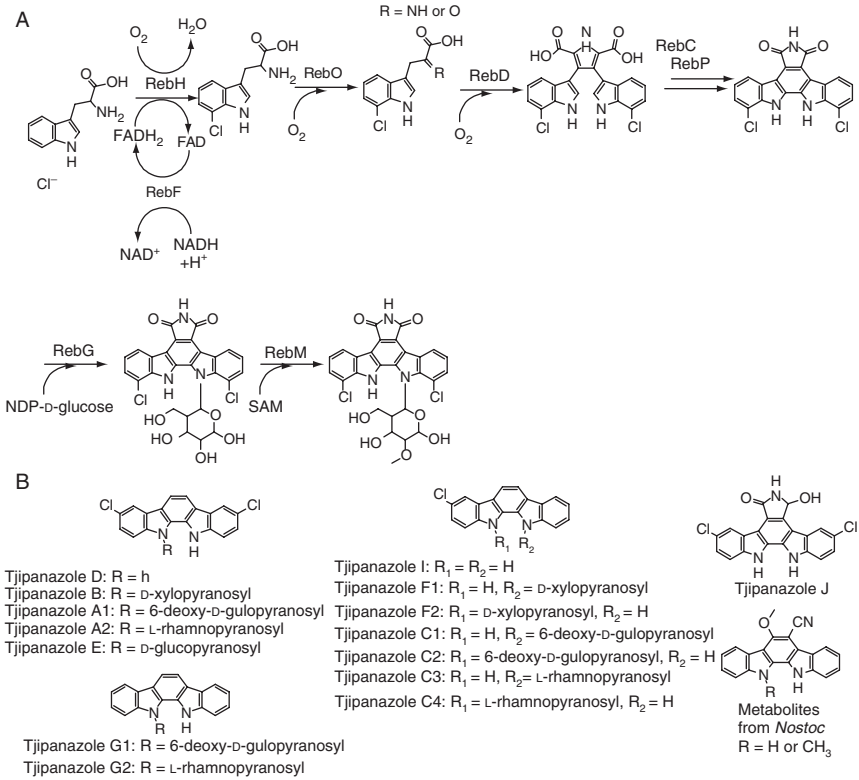


FIG. 37. The tjipanazoles. (A) Biosynthesis of the actinomycete alkaloid, rebeccamycin. (B) Examples of tjipanazoles from cyanobacteria.

The structure of tjipanazole J (Fig. 37B) strongly suggests that the biosynthesis of the tjipanazoles is similar to that of rebeccamycin. The uppermost succinimidyl ring may undergo ring opening and hydrolysis followed by reductive decarboxylation of both sites in the tjipanazoles. Several tjipanazole glycosides have been observed suggesting broad specificity of the glycosyl transferase or downstream modification of the sugar moiety. Chlorination in the tjipanazoles occurs at the most electrophilic sites of the aryl ring which is the apparent mechanism of chlorination in rebeccamycin (Yeh *et al.*, 2005, 2006). Some related compounds have been reported from *Nostoc* (Knubel *et al.*, 1990). These compounds were reported to have antiviral activity.

Some additional indole-based carbazoles have been reported that contain only one indole moiety. Hyellazole (Fig. 38A) was isolated from *Hyella* (Cardellina *et al.*, 1979a). The carbazole is part of a biphenyl moiety. Also, a series of dihalogenated carbazoles (Fig. 38B) have been isolated from *Kyrtuthrix* (Lee *et al.*, 1999b).

A small family of chlorinated β-carbolines, the bauerines (Fig. 38C), has been reported from *Dichothrix* (Larsen *et al.*, 1994). The β-carbolines are typically synthesized by Pictet–Spengler cyclization of tryptamine derivatives with carbonyl compounds. In the case of the bauerines, the carbonyl moiety is most likely contributed by formate or a related compound in the C_1 pool.

a. *Indole/Isoprenoid Hybrids.* There are many alkaloids found in cyanobacteria that result from the combination of aromatic amino acids with isoprenoid units. In most cases, the isoprenoid portion undergoes

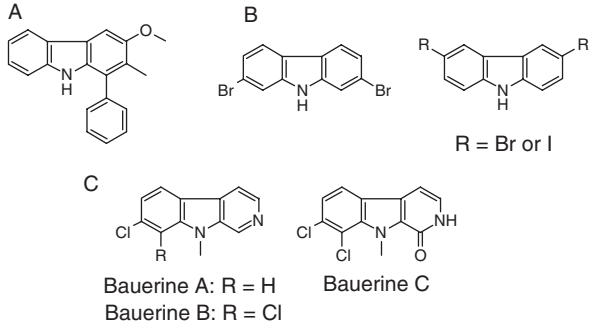


FIG. 38. Carbazole and β-carboline metabolites from cyanobacteria. (A) Hyellazole. (B) Halogenated carbazoles from *Kyrtuthrix*. (C) Bauerines.

cyclization to form more complex structures. The amino acid portion is always tryptophan derived.

One of the first reported isoprenoid/tryptophan structures from cyanobacteria was that of lyngbyatoxin from *Lyngbya* (Fig. 39) (Aimi *et al.*, 1990; Cardellina *et al.*, 1979c; Sakai *et al.*, 1986). This compound is a causative agent of seaweed dermatitis and a potent tumor promoter on skin (Fujiki *et al.*, 1981), and a potent activator of protein kinase C (Fujiki *et al.*, 1981; Moore *et al.*, 1986a; Nakayasu *et al.*, 1981; Robinson *et al.*, 1991). Lyngbyatoxins are structurally very similar to the teleocidins from *Streptomyces* (Hagiwara *et al.*, 1985; Hitotsuyanagi *et al.*, 1984; Irie *et al.*, 1987, 1994; Nakae *et al.*, 2006; Nakata *et al.*, 1966; Takashima and Sakai, 1960; Takashima *et al.*, 1962), differing primarily by the absence of cyclization of the isoprenoid chain. Here again, an unusual co-occurrence of cyanobacterial and actinomycete metabolites is seen. The gene cluster responsible for the production of lyngbyatoxin has been cloned (Edwards and Gerwick, 2004). It was possible to achieve heterologous expression of *ltxC*, the gene responsible for the condensation of an indolactam intermediate with geranyl pyrophosphate. HPLC/MS analysis of reactions containing the purified protein LtxC and the two substrates indicated successful production of lyngbyatoxin. Formation of the correct chiral configuration was confirmed by circular dichroism. A proposed biosynthesis of the lyngbyatoxins is shown in Fig. 39 (Edwards and Gerwick, 2004).

Several polycyclic tryptophan/isoprenoid compounds have been reported from *Fischerella*, *Hapalosiphon*, *Westiella*, and *Westiellopsis*. The rich diversity in their cyclic structures highlights the flexibility of carbocation-based isoprenoid chemistry in forming carbon–carbon bonds. A scheme presenting possible biogenetic relationships among some of the dozens of reported compounds in this family is shown in Fig. 40A (Stratmann *et al.*, 1994c). In addition to the structures shown, there are other modifications involving hydroxylation, changes in the numbers of double bonds, and even the introduction of additional rings through heterocyclization. Also not shown is the diversity in stereochemistry among the compounds; epimers for many (but not all) of the stereocenters have been described. The hapalindoles (Fig. 40B) (Klein *et al.*, 1995; Moore *et al.*, 1984b, 1987a; Polchai *et al.*, 2003; Stratmann *et al.*, 1994c) appear to result from regiospecific condensation of a tryptophan derivative to an analogue of geranyl pyrophosphate. In some hapalindoles (e.g., hapalindoles A and B), additional cyclization of an isoprenoid carbon to a benzenoid carbon leads to formation of a fourth ring. Oxidation of the pyrrole ring of the indole leads to lactam derivatives such as the anhydrohapaloxindoles (Moore *et al.*, 1987b, 1989b)

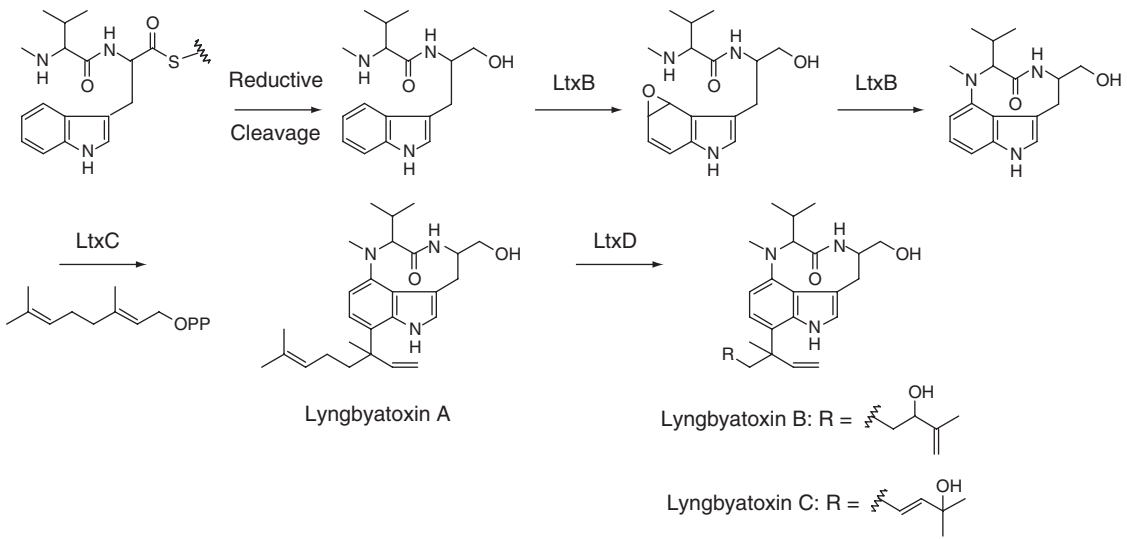


FIG. 39. Proposed biosynthesis of the lyngbyatoxins.

and products of additional cyclization such as the hapalindolinones (Klein *et al.*, 1995; Schwartz *et al.*, 1987). Oxidative ring opening of hapalindoles leads to hapalonamides and fontonamides (Moore *et al.*, 1987b, 1989b). The anhydrohapaloxindoles and hapalonamides can be produced synthetically by singlet oxygen oxidation of hapalindoles

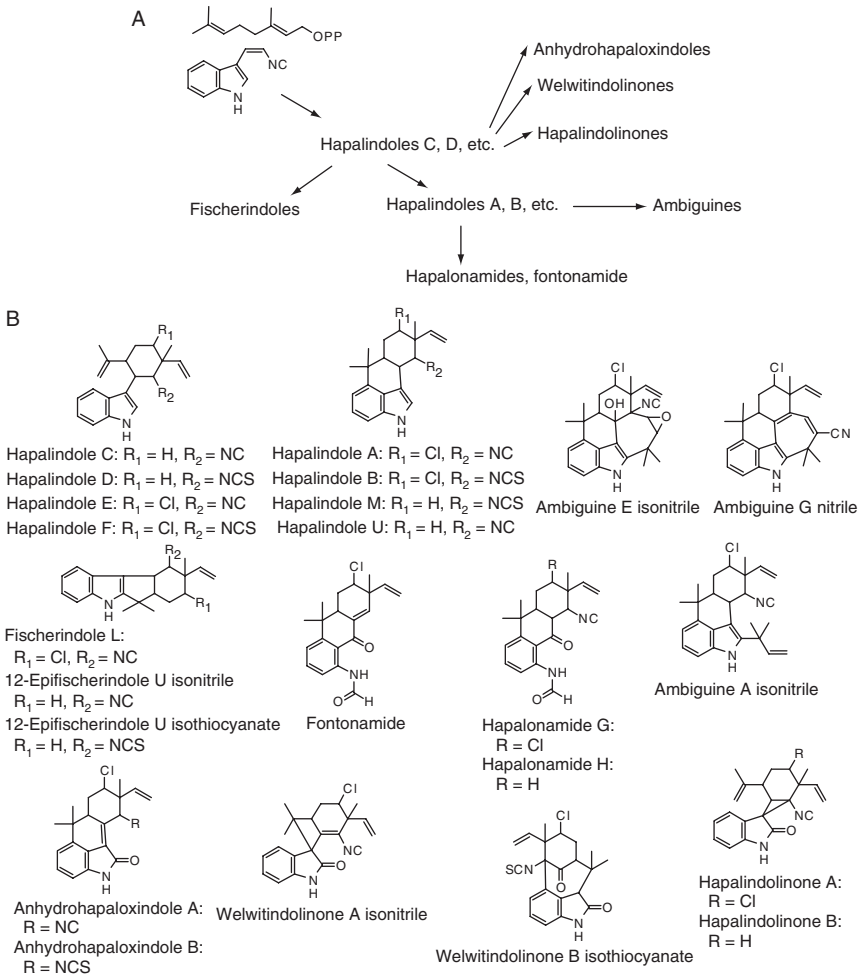


FIG. 40. Several indole/isoprenoid alkaloids from cyanobacteria. (A) Proposed biogenetic relationships among apparently related indole/isoprenoid alkaloids. (B) Examples of the hapalindoles and related alkaloids.

(Moore *et al.*, 1987b). An alternative ring formation of the lactam derivative followed by a rearrangement can give rise to the welwitindolinones (Jimenez *et al.*, 1999; Stratmann *et al.*, 1994c). Alternatively, an additional isoprene unit can be attached to the pyrrole of the tetracyclic hapalindoles and undergo cyclization to give the ambiguines (Huber *et al.*, 1998; Smitka *et al.*, 1992). Ambiguine G nitrile appears to result from a rearrangement in which the isonitrile migrates and reverses polarity to give a nitrile. Several members of this family include seven-membered rings. An alternative cyclization pathway is possible for tricyclic hapalindoles (e.g., hapalindoles C and D) that leads to the cyclopentane-containing fischerindoles (Park *et al.*, 1992; Stratmann *et al.*, 1994c). Isotopic feeding studies exploring the origin of the isonitrile group in the hapalindoles indicated that the cellular C_1 pool contributes to the carbon of the isonitrile group (Bornemann *et al.*, 1988). These studies also showed some intact incorporation of N and C2 of glycine into the isonitrile group, again implicating the tetrahydrofolate C_1 pool. The hapalindoles are antibiotic and found to inhibit RNA synthesis in bacteria (Doan *et al.*, 2000, 2001). The welwitindolinones were found to reverse multiple drug resistance in cancer cells (Smith *et al.*, 1995) and to interfere with microtubule formation (Zhang and Smith, 1996).

6. *Other Aromatic Alkaloids*

Two types of quinone alkaloids have been reported (Fig. 41A). Aulosirazole from *Aulosira* (Stratmann *et al.*, 1994a), contains an isothiazole ring, a rare occurrence among natural compounds. Two potential antimalarial compounds, calothrixins A and B, were isolated from *Calothrix* (Rickards *et al.*, 1999). The original study suggested a pathway that involves ring opening of an indolo[2,3*a*]carbazole compound similar to a carbazole reported from *Nostoc* (Fig. 37B) followed by ring closure to give the calothrixins. In addition to their activity against *Plasmodium*, the calothrixins inhibit bacterial RNA synthesis (Doan *et al.*, 2000, 2001), and induce apoptosis in human cancer cells (Chen *et al.*, 2003), presumably through their capacity to undergo oxidative cycling (Bernardo *et al.*, 2004).

Louludinium, a bicyclic pyridinium-containing compound (Fig. 41B), was reported from *Lyngbya* (Yoshida and Scheuer, 1998). A similar compound, montipyridine, has been reported from a stony coral (Alam *et al.*, 2001).

The highly nitrogenated heterocycle, nostocine A (Fig. 41C), was reported from *Nostoc* (Hirata *et al.*, 1996). The violet pigment is toxic

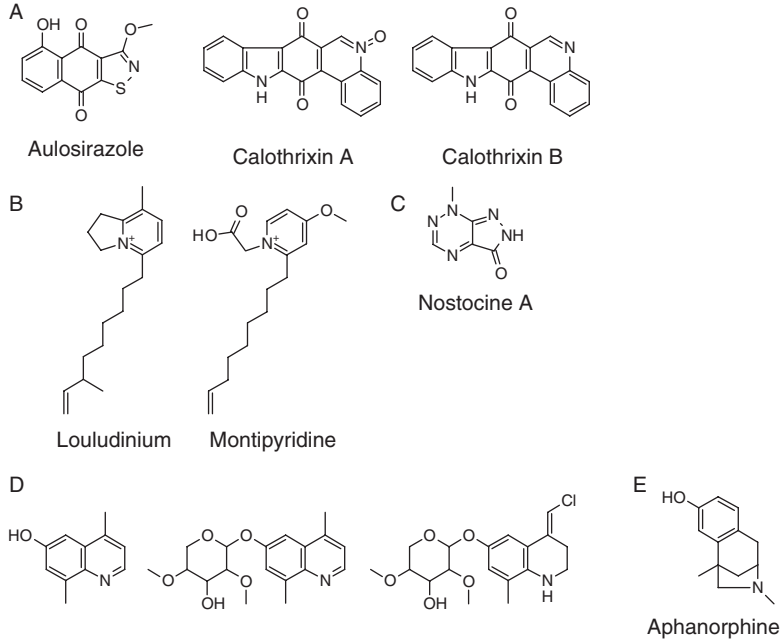


FIG. 41. Various aromatic alkaloids from cyanobacteria. (A) Quinone alkaloids. (B) Louludinium from *Lyngbya* and a similar compound from stony coral. (C) Nostocine A. (D) Quinoline alkaloids from *Lyngbya*. (E) Aphanorphine.

against several organisms (Hirata *et al.*, 2003), apparently through the generation of reactive oxygen species such as superoxide radical anion when in the presence of reducing agents (Hirata *et al.*, 2004). Production of nostocine A in *Nostoc* is enhanced when the cells are exposed to oxidative stress (Hirata *et al.*, 2003), suggesting a possible role for a reduced form of nostocine A as a protectant against oxidative conditions.

A small number of quinoline alkaloids (Fig. 41D) have been reported from *Lyngbya*. In the first two reported examples (Orjala and Gerwick, 1997), the quinoline system is fully aromatized and the substituent at position four is a methyl group. The third example (Nogle and Gerwick, 2003) contains a chloroalkene substituent at position four, yet another occurrence of this functionality. Nogle and Gerwick (2003) proposed a mixed PKS/NRPS biosynthesis of the latter compound in which the piperidine ring derives from a β-alanine starter unit and the phenolic ring derives from three acetate extender units.

The bicyclic compound aphanorphine was reported from *Aphanizomenon* (Gulavita *et al.*, 1988). This compound could result from multiple cyclizations of a tyrosine derivative and has inspired many efforts at total synthesis (Bower *et al.*, 2005; ElAzab *et al.*, 2002; Fadel and Arzel, 1997; Fuchs and Funk, 2001; Honda *et al.*, 1992; Hu and Zhai, 2003; Katoh *et al.*, 2005; Kita *et al.*, 2003; Meyers *et al.*, 1995; Node *et al.*, 1996; Shimizu *et al.*, 1997; Takano *et al.*, 1990; Tamura *et al.*, 2001, 2003; Tanaka *et al.*, 2001; Zhai *et al.*, 2003).

7. *Nucleosides*

The cytotoxic, antimycotic 7-deazapurine nucleosides tubercidin and toyocamycin and their 5′-α-D-glucopyranoside conjugates have been reported from *Tolypothrix* and *Plectonema* (Stewart *et al.*, 1988). Both tubercidin (Anzai *et al.*, 1957) and toyocamycin (Nishimura *et al.*, 1956) have been previously reported from actinomycetes. Radiotracer feeding studies of adenosine analogues have been performed on tubercidin (Smulson and Suhadolnik, 1967) and on toyocamycin (Suhadolnik and Uematsu, 1970; Uematsu and Suhadolnik, 1970) to probe the biosynthetic pathways leading to their production in actinomycetes. The biogenetic origin of the pyrrole carbons suggested by those studies is shown in Fig. 42A. Derivatives of toyocamycin and tubercidin have been reported from sponges (Biabani *et al.*, 2002; Zabriskie and Ireland, 1989) and tunicates (Mitchell *et al.*, 1996). A C-glycoside nucleoside, 9-deazaadenosine, and its 5′-α-D-glucopyranoside conjugate (Fig. 42B) were isolated from *Anabaena* (Namikoshi *et al.*, 1993).

8. *Guanidine-Containing Alkaloids*

Cyanobacteria are associated with several neurotoxic guanidine-containing alkaloids. Some of the toxins have been previously identified from other unrelated organisms. Fresh water species of the genus *Lyngbya* were found to contain several analogues of the highly potent paralytic toxins gonyautoxin and saxitoxin (Fig. 43A and B) (Carmichael *et al.*, 1997; Onodera *et al.*, 1997), both classes of which are produced by dinoflagellates in the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium* (Bordner *et al.*, 1975; Llewellyn, 2006; Schantz *et al.*, 1966, 1975; Shimizu *et al.*, 1976). Saxitoxin and its analogues have long been known to antagonize voltage-gated sodium channels. Studies have uncovered several other potential targets, not all of them ion channels (Llewellyn, 2006). In spite of the many difficulties involved in precursor-feeding studies in dinoflagellates, much has been learned about the biosynthesis of saxitoxin and its analogues by examining the biosynthesis in producing

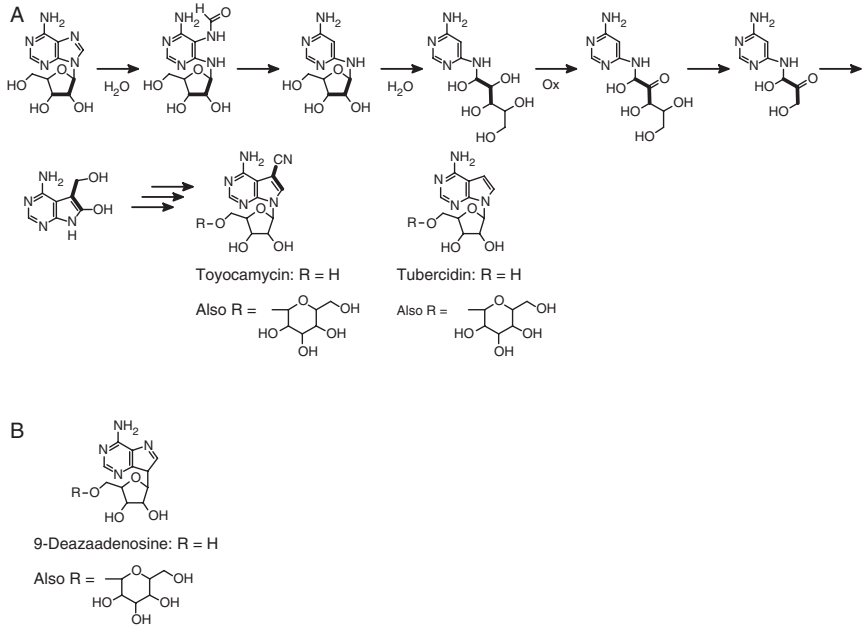


FIG. 42. Nucleoside analogues from cyanobacteria. (A) A possible biogenetic origin of pyrrole carbons in toyocamycin and tubercidin in actinomycetes based on labeling studies. The bold bonds indicate observed incorporation of labeled ribose carbons in the pyrrole. (B) 9-Deazaadenosine derivatives.

cyanobacteria (Gupta *et al.*, 1989; Shimizu, 1986; Shimizu *et al.*, 1984). The formation of the tricyclic nucleus is unusual in that arginine is condensed with an acetate starter unit via the α-carbon rather than through the amine group (Fig. 43A). The carboxyl group of arginine is lost and the primary amine of arginine is incorporated in formation of one of the guanidinium groups. The side chain carbon is derived from SAM. The co-occurrence of saxitoxin-like compounds in both dinoflagellates and cyanobacteria raises the question of how this distribution occurred. A study has provided preliminary evidence, suggesting that production of saxitoxin in *Anabaena* strains may be associated with LGT events (Pomati and Neilan, 2004).

The hepatotoxic guanidine alkaloid cylindrospermopsin was originally isolated from *Cylindrospermopsis* (Ohtani *et al.*, 1992), but cylindrospermopsin or its 7-epi (Banker *et al.*, 2000) and 7-deoxy (Norris *et al.*, 1999) analogues (Fig. 43C) have since been reported from various

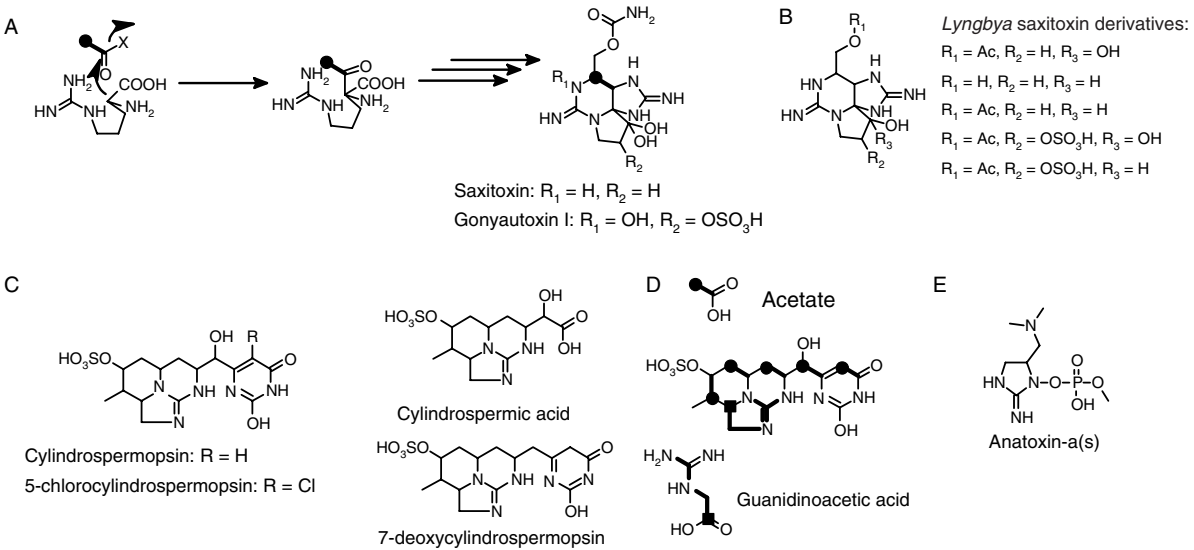


FIG. 43. Guanidine-containing alkaloids from cyanobacteria. (A) Biogenesis of saxitoxin and gonyautoxin. (B) Various saxitoxin derivatives from *Lyngbya*. (C) Cylindrospermopsin and related compounds. (D) Biogenetic origin of carbon atoms in cylindrospermopsin. (E) Anatoxin-a(s).

genera, including *Umezakia* (Harada *et al.*, 1994), *Aphanizomenon* (Banker *et al.*, 1997, 2000; Preuβel *et al.*, 2006), and *Raphidiopsis* (Li *et al.*, 2001). Potential contamination of water supplies with cylindrospermopsin presents a major risk for human health (Falconer and Humpage, 2005, 2006). Cylindrospermopsin cannot be removed by filtration of water supplies because it is highly polar and often leaks from cyanobacteria extensively (Shaw *et al.*, 1999). Blooms of producing organisms can be difficult to detect because some of the organisms do not grow as surface scums as do other cyanobacteria implicated in human poisonings, for example *Microcystis*. Cylindrospermopsin is also very potent and causes toxic effects in several organs (Hawkins *et al.*, 1985). It can take several days after exposure for the toxic effects to occur. In addition there are concerns that chronic subtoxic doses can be carcinogenic (Falconer and Humpage, 2001). Two nontoxic analogues 5-chlorocylindrospermopsin and cylindrospermic acid have been produced semisynthetically, suggesting that the uracil moiety is required for toxicity (Banker *et al.*, 2001). Feeding experiments with stable-isotope-labeled precursors indicated that cylindrospermopsin results from a mixed PKS/NRPS pathway with a guanidinoacetic acid starter unit and five acetate extenders (Fig. 43D) (Burgoyne *et al.*, 2000). It was found that glycine can also be incorporated intact as a starter unit, but the guanidine group was not labeled on feeding with labeled arginine precursors. A gene cluster potentially involved in cylindrospermopsin biosynthesis has been cloned (Schembri *et al.*, 2001; Shalev-Alon *et al.*, 2002). The most intriguing aspect of the cloned pathway is the presence of a gene that shows homology to guanidinyl transferases. Additionally, a gene encoding sequential NRPS and PKS modules, and an additional gene encoding a PKS have been identified. However, the number of modules identified so far do not account for the number of acetate groups present in cylindrospermopsin unless the PKS module is acting iteratively. In addition, no putative enzymes involved in the other modification steps such as *C*-methylation, sulfation, hydroxylation, and multiple cyclizations have been identified within the cluster. Comparison of gene cluster sequences among distantly related toxin producers suggested that the guanidinyl transferase gene may have been involved in LGT (Kellmann *et al.*, 2006).

Anatoxin-a(s) (Fig. 43E), an unusual guanidinyl phosphoester that irreversibly inhibits acetylcholinesterase (Hyde and Carmichael, 1991), is a toxin produced by *Anabaena* (Mahmood and Carmichael, 1986, 1987; Matsunaga *et al.*, 1989). Anatoxin-a(s) decomposes readily in alkaline conditions but is significantly more stable at neutral or acidic

pH (Matsunaga *et al.*, 1989). Labeled precursor feeding studies established arginine as a precursor to anatoxin-a(s) (Moore *et al.*, 1992b) via 4-hydroxyarginine (Hemscheidt *et al.*, 1995).

9. *Anatoxin-a*

Anatoxin-a from *Anabaena* (Devlin *et al.*, 1977) and homoanatoxin-a from *Oscillatoria* (Skulberg *et al.*, 1992), like anatoxin-a(s), are neurotoxins acting at the neuromuscular junction, but are unrelated in both structure and mechanism of action to anatoxin-a(s). Both anatoxin-a and homoanatoxin-a are small bicyclic agonists of nicotinic acetylcholine receptor ion channels (Aronstam and Witkop, 1981; Wonnacott *et al.*, 1992). There have been conflicting data on the biosynthesis of anatoxin-a. One set of studies showed incorporation of radioisotopically labeled arginine, ornithine, and putrescine into anatoxin-a (Gallon *et al.*, 1990, 1994). Also, radiolabeled Δ^1-pyrroline was incorporated into anatoxin-a and fractionation of cell-free extracts produced ornithine/arginine decarboxylase and diamine oxidase activities that could lead to the formation of Δ^1-pyrroline (Fig. 44A). These studies also showed that toxic strains of *Anabaena* contained plasmids, whereas a nontoxic strain did not, that a nontoxic strain could be made toxic by transformation with DNA from a toxic strain, and that loss of toxin production in a strain was associated with a change in plasmid size from 10 to 6.5 kb. If toxin production is encoded on a plasmid, this could provide a mechanism for LGT of the toxin biosynthetic genes.

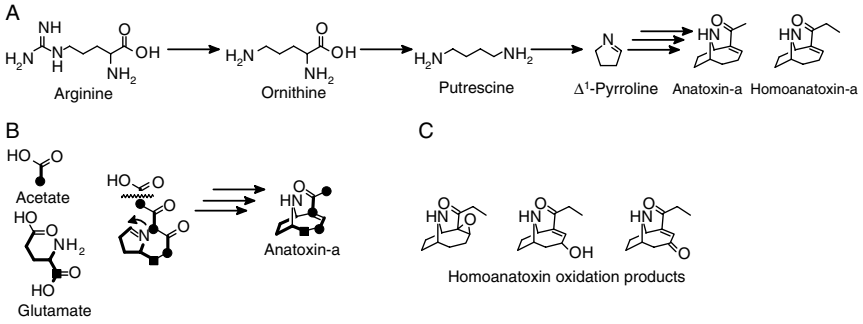


FIG. 44. Anatoxin and homoanatoxin. (A) A proposed biogenetic scheme based on one set of incorporation experiments. (B) A second biogenetic scheme based on another set of incorporation experiments. (C) Oxidized products of homoanatoxin observed in cyanobacteria.

In contrast, another study showed the carbon skeleton of anatoxin-a to be composed of acetate and glutamate, and that C1 of glutamate is retained in anatoxin-a (Fig. 44B) (Hemscheidt *et al.*, 1995). The two sets of studies are at odds because Δ^1-pyrroline cannot be produced from glutamate without loss of C1, suggesting that incorporation of Δ^1-pyrroline into anatoxin can take place only after carboxylation of the pyrroline ring. A study reported that the extra carbon in homoanatoxin-a comes from SAM (Namikoshi *et al.*, 2004). Oxidation products of homoanatoxin have also been reported (Fig. 44C) (Namikoshi *et al.*, 2003, 2004).

VI. Isoprenoids

Isoprenoids are a large structural class that includes terpenoids, steroids, and carotenoids. They are produced by condensation of multiple C_5 units based on isopentenyl pyrophosphate (IPP) or dimethylallyl pyrophosphate (DMAP) to form long branched chains that can subsequently undergo multiple cyclizations and bond migrations. The complexity observed in many isoprenoid products is a direct result of the carbocation chemistry that enzymes exploit for their cyclizations. Carbocations are highly reactive intermediates that readily undergo carbon–carbon bond formation and migration of branched atoms from one carbon to another.

There are fundamental differences in the way that isoprenoid precursors are synthesized in different organisms (Dewick, 2002). Until about the mid 1990s, the major pathway was believed to be the mevalonate pathway, which is the dominant pathway in archaea, eukaryotes, the cytosol of plants, and in some eubacteria (Dewick, 2002; Kuzuyama, 2002). The mevalonate pathway uses acetate to produce IPP and DMAP by way of 3-hydroxy-3-methylglutaryl-CoA (HMG) (Fig. 45A). A second major pathway is present in most eubacteria and in the plastids of photosynthetic organisms (Dewick, 2002; Kuzuyama, 2002). The second pathway has been referred to by many names, most prominently the deoxyxylulose phosphate (DXP) pathway and the methylerythritol phosphate (MEP) pathway (Fig. 45B). The latter usage has been recommended because of the status of MEP as the first committed metabolite unique to the pathway (Dewick, 2002). Enzymes from the MEP pathway have been cloned from the cyanobacterium *Synechococcus* (Miller *et al.*, 1999, 2000). There is evidence that IPP and DMAP do not interconvert in *Synechococcus*, suggesting that each is synthesized separately (Dewick, 2002).

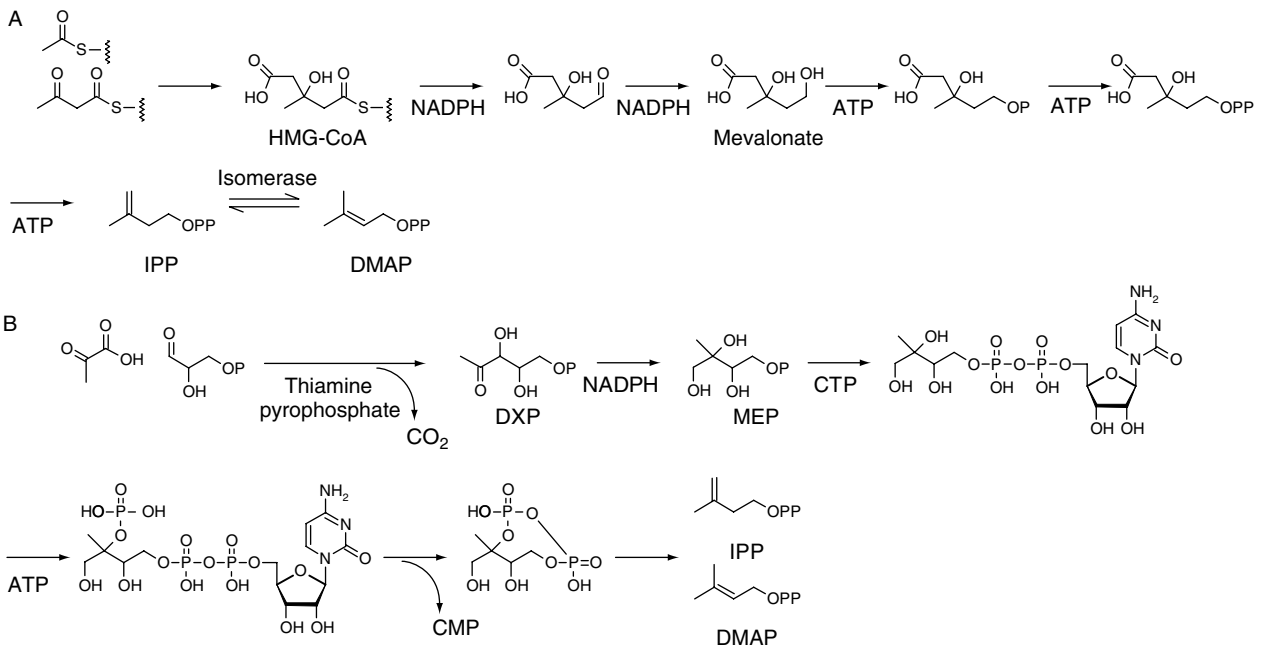


FIG. 45. Major pathways for isoprenoid precursor biosynthesis. (A) The mevalonate pathway, which predominates in eukaryotes, archaea, and some eubacteria. (B) The MEP pathway, which predominates in many eubacteria and is present in cyanobacteria.

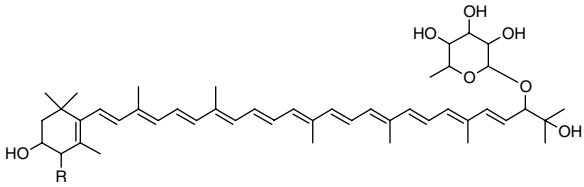
A. Carotenoids

Glycosylated carotenoids are characteristic pigments in cyanobacteria. While glycosylated pigments are found in other groups or organisms, the position of attachment of the sugar is unique to cyanobacterial carotenoids (Fig. 46). The most common carotenoid is the L-quinovose-conjugated myxoxanthophyll (Foss *et al.*, 1986; Heilbron and Lythgoe, 1936; Hertzberg and Liaaen-Jensen, 1969). Closely related compounds include aphanizophyll (Hertzberg and Liaaen-Jensen, 1971; Tischer, 1938) and 4-ketomyxoxanthophyll (Francis *et al.*, 1970; Hertzberg *et al.*, 1971), both of which contain L-rhamnose as the sugar. Oscillaxanthin (Foss *et al.*, 1986; Hertzberg and Liaaen-Jensen, 1969; Karrer and Rutschmann, 1944) and P 496 (Francis *et al.*, 1970) are both diglycosides of acyclic carotenoids. Both quinovose and rhamnose have been reported in oscillaxanthin. There does seem to be some flexibility in the stereochemistry of the carbohydrate moieties between species (Takaichi *et al.*, 2001). The function of the glycosylated carotenoids is not known but studies indicate that they provide stabilization of the thylakoid membrane (Mohamed *et al.*, 2005) and protection against oxidative damage (Maeda *et al.*, 2005; Schäfer *et al.*, 2005). Nonglycosylated carotenoids include nostoxanthin and caloxanthin (Buchecker *et al.*, 1976).

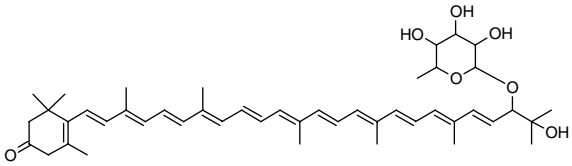
B. Terpenoids

Apart from the carotenoid metabolites, there have been relatively few isoprenoids reported from cyanobacteria. *Microcystis* produces the monoterpenoid β-cyclocitral (Fig. 47A) (Jüttner, 1976), a volatile compound that has an effect on the flavor and odor of bodies of water in which *Microcystis* blooms occur (Zimba and Grimm, 2003).

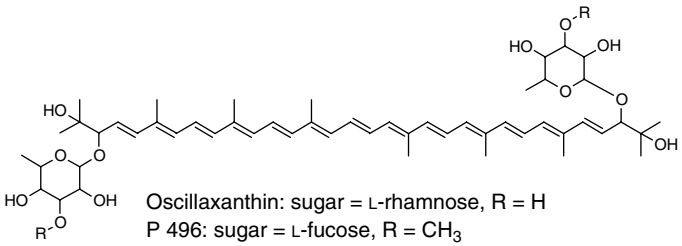
Several meroterpenoids (terpenoids incorporated with intermediates of other biosynthetic pathways) have been isolated from *Tolypothrix* and *Nostoc* (Fig. 47C). Tolypodiol was isolated from *Tolypothrix* (Prinsep *et al.*, 1996). All other meroterpenoids reported thus far come from *Nostoc* (Jaki *et al.*, 1999, 2000a,b). In all cases, the compounds appear to result from electrophilic aromatic substitution of *p*-hydroxybenzoic acid, likely derived from the shikimate pathway, by a linear diterpene precursor. Formation of an ether bond at C13 completes the structure. An outline of a possible biosynthetic route is shown for tolypodiol (Fig. 47B). The structure of noscomin, which lacks the ether ring, supports this putative biosynthetic pathway, as do the comnostins. This latter group features an alternative cyclization step, producing a cyclopentane ring at one end, and an additional methyl migration step. Oxidation of one of



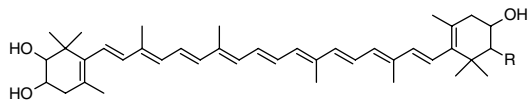
Myxoxanthophyll: sugar = L-quinovose, R = H
Aphanizophyll: sugar = L-rhamnose, R = OH



4-Ketomyxoxanthophyll: sugar = L-rhamnose



Oscillaxanthin: sugar = L-rhamnose, R = H
P 496: sugar = L-fucose, R = CH_3



Nostoxanthin: R = OH
Caloxanthin: R = H

FIG. 46. Carotenoids from cyanobacteria.

the *gem*-dimethyl groups adds further structural diversity. Another meroterpenoid from *Nostoc* undergoes similar methyl migration and ether linkage at C7 to form a seven-membered ring. The diterpenoid skeletons of the *Nostoc* compounds were originally reported as having no known precedent (Jaki *et al.*, 1999, 2000b).

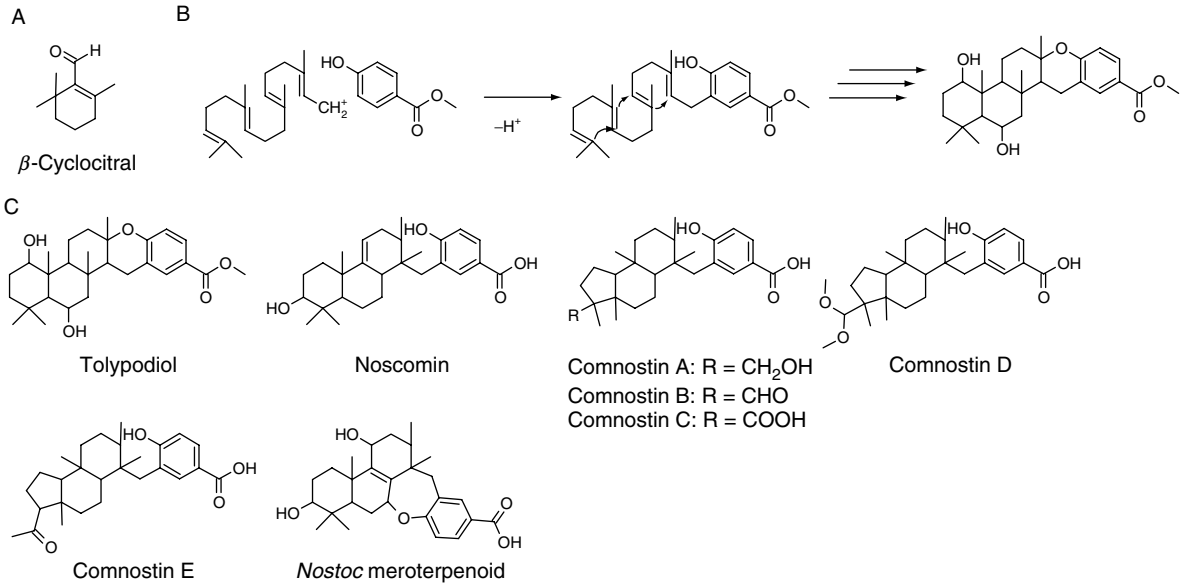
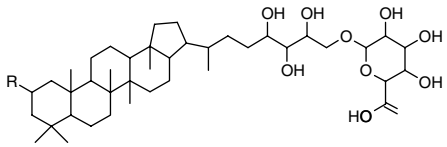
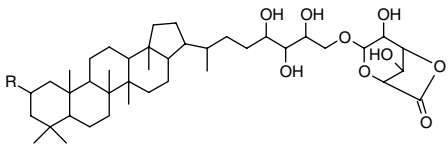


FIG. 47. Various terpenoids from cyanobacteria. (A) β-Cyclocitral. (B) Meroterpenoids from cyanobacteria.

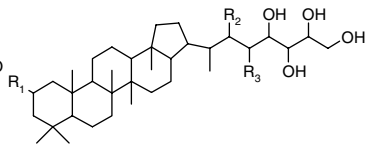
Several bacteriohopanepolyols (C_{35} terpenoids) have been reported from *Synechococcus* (Llopiz *et al.*, 1996), *Prochlorothrix* (Simonin *et al.*, 1996), and *Nostoc* (Zhao *et al.*, 1996). All contain a hopane nucleus with a polyhydroxy side chain derived by formation of a carbon–carbon bond between a hopane precursor and a pentose sugar (Fig. 48) (Flesch and Rohmer, 1988). In *Prochlorothrix* and *Synechococcus*, galacturonyl conjugates of the bacteriohopanepolyols were reported. In *Prochlorothrix*, the compounds were localized primarily in the cell wall and thylakoid membrane, supporting a role for these compounds as membrane stabilizers (Simonin *et al.*, 1996). In all of the compounds reported, a mixture was obtained of the C2-methyl and *des*-methyl analogues. Methylation at C2 of hopanoids occurring in sediments has been proposed as a marker of cyanobacteria in geological samples because of the wide distribution of this modification within cyanobacteria, the rarity of it in other organisms, and the enhanced preservation of bacteriohopanepolyols through incorporation into the kerogen matrix via their multiple hydroxyl groups (Summons *et al.*, 1999).



Synechococcus:
sugar = glucuronosyl or galacturonosyl
R = H or CH_3
Prochlorothrix:
sugar = galacturonosyl or altruronosyl
R = H or CH_3



Prochlorothrix:
R = H or CH_3



Nostoc:
R_1 = H or CH_3
R_2 = H, R_3 = OH
R_2 = OH, R_3 = H

FIG. 48. Hopanoids from cyanobacteria.

VII. Other Cyanobacterial Metabolites

This section collects a number of compounds that were not easily classified into any of the other sections. It includes a soluble protein with interesting pharmacological activity, a family of butyrolactone derivatives of unknown biogenesis, and some small molecules apparently derived from citrate. Some phenylpropanoids are also described.

A. Proteins

One of the more interesting developments in the isolation of biologically active molecules from cyanobacteria was the discovery of soluble proteins with potent anti-HIV activity. Cyanovirin-N was isolated from aqueous extracts of *Nostoc* (Boyd *et al.*, 1997; Gustafson *et al.*, 1997). The protein contains 101 amino acid residues and consists of two repeat units, each about 50 residues long and showing >50% similarity to one another. Cyanovirin-N contains four cysteine residues that form two intrachain disulfide bridges. The three-dimensional structure of cyanovirin-N was determined by NMR (Bewley *et al.*, 1998) and by X-ray crystallography (Yang *et al.*, 1999). Cyanovirin-N is composed primarily of β-sheets and has a pseudosymmetric axis of rotation that relates the two repeat units, which interact with one another and do not form separate domains. The shape is oblate. A surprising observation in the X-ray structure was the phenomenon of domain swapping in which a dimer of cyanovirin-N was formed where each repeat unit interacted with the opposite repeat unit of the other monomer. Dimerization of cyanovirin-N is a complex phenomenon that cannot be entirely explained by pH effects (Botos and Wlodawer, 2003). Cyanovirin-N appears to block HIV infection and transmission by binding to the glycoprotein gp120 of the viral envelope and preventing its interaction with cell membrane receptors required for fusion (Boyd *et al.*, 1997). A particularly promising aspect of its mechanism of action is that cyanovirin-N remains active against several mutant strains that are otherwise resistant to other agents that block interactions between gp120 and the cellular surface receptor CD4. Studies show that cyanovirin-N binds with high affinity to mannose-rich oligosaccharides, which can be found in gp120 (Bolmstedt *et al.*, 2001). A related protein, scytovirin from *Scytonema*, is also active against HIV (Bokesch *et al.*, 2003). A study indicates that expression of the N-terminal half of scytovirin produces a protein that retains anti-HIV activity (Xiong *et al.*, 2006). Another interesting development has been the isolation of a protein microvirin from *Microcystis* that shows similar

carbohydrate-binding properties to, and sequence similarity with, cyanovirin-N (Kehr *et al.*, 2006). The latter study included intriguing observations of potential interactions between microvirin and the toxin microcystin. Also, microvirin shows affinity to components of the lipopolysaccharide fraction of *Microcystis*. These results suggest that the microvirins may be involved in intraspecies cell–cell recognition and/or aggregation and that this behavior may somehow involve microcystin.

B. Aromatic Compounds

Several halogenated aromatic compounds have been reported from cyanobacteria (Fig. 49A), and are most likely derived from the shikimate metabolic pathway. 4-Benzyl-2-chlorophenol was reported from *Anacystis* (Gribble, 1996). A polybrominated aldehyde and its alcohol reduction product were reported from *Calothrix* (Pedersen and DaSilva, 1973). The ambigols are chlorinated polyphenols isolated from *Fischerella* (Falch *et al.*, 1993; Wright *et al.*, 2005) that bear resemblance to the phlorotannins of brown algae. The pattern of chlorination of the ambigols and their co-occurrence with tjipanazole D (Section V.B.5) suggest that halogen is introduced via transferases which employ an electrophilic aromatic substitution mechanism.

A simple anthraquinone analogue (Fig. 49B) has been reported from *Nostoc* (Jaki *et al.*, 2000b). There are multiple paths by which an anthraquinone can be made, including a nonreducing polyketide synthase as seen in fungi, or by condensation of two shikimate products. There are no features of the reported structure that can exclude either possibility.

1. *Phenylpropanoids*

Two indanones have been reported from cyanobacteria (Fig. 49C). One indanone from *Lyngbya* contains an aldehyde functionality and was reported to inhibit human VEGF promoter (Nagle *et al.*, 2000). Another was reported from *Nostoc* (Jaki *et al.*, 2000a). These compounds could potentially be formed by cyclization of a phenylpropanoid precursor.

The maculalactones (Fig. 50) are a family of phenylpropanoids isolated from *Kyrtuthrix* (Lee *et al.*, 1998, 1999b; Tsui *et al.*, 1996; Wong *et al.*, 2002). Formulating a plausible biogenesis for the two main maculalactone nucleus structures in the absence of precursor labeling studies poses some interesting challenges. First, consider the simpler triaryl monocyclic nucleus (e.g., maculalactone A). There appears to be a clear phenylpropanoid unit involving the acyl carbon. If the two remaining aromatic rings also come from phenylpropanoid precursors, then two

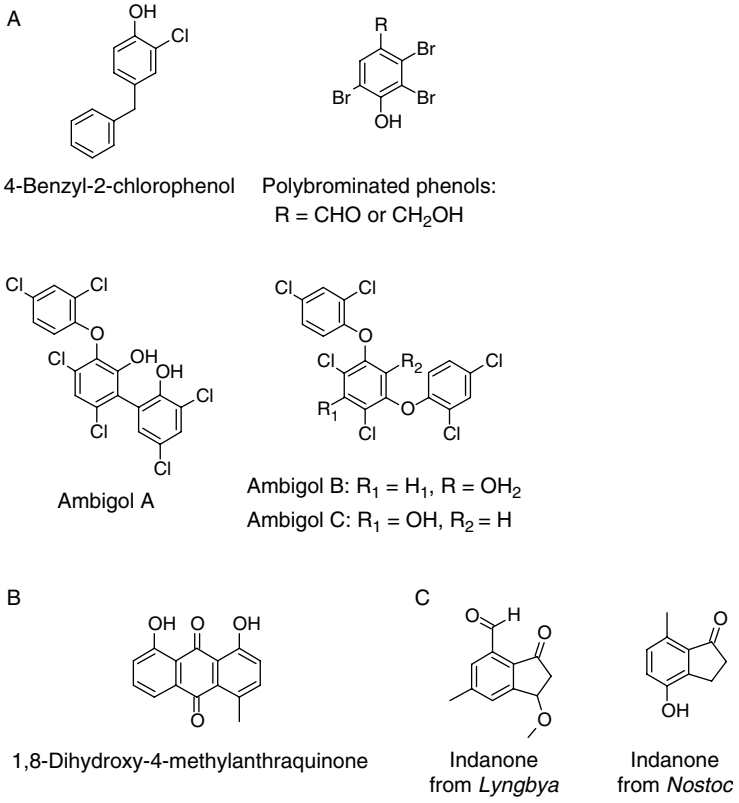


FIG. 49. Various aromatic metabolites from cyanobacteria. (A) Halogenated phenols. (B) An anthraquinone from cyanobacteria. (C) Indanones from cyanobacteria.

carbons must be lost from them, but it is not clear which of, or how, these carbons are lost. In a second structure type, it appears that a fourth phenylpropanoid ring is condensed with the first nucleus structure by the formation of two new carbon–carbon bonds to two different carbons. The large number of alcohol and ether groups suggests that epoxidation of double bonds is prevalent. Maculalactone M appears to be an oxidative ring cleavage product (Wong *et al.*, 2002). The algicidal and chlorinated nostoclides, isolated from *Nostoc* (Yang *et al.*, 1993) and cyanobacterins, isolated from *Scytonema* (Lee and Gleason, 1994; Mason *et al.*, 1982; Pignatello *et al.*, 1983), appear to derive from similar pathways. In the latter two classes of compounds, however, the aromatic rings are hydroxylated and a derivative of isovaleric acid or of valine appears to take the place of a phenylpropanoid.

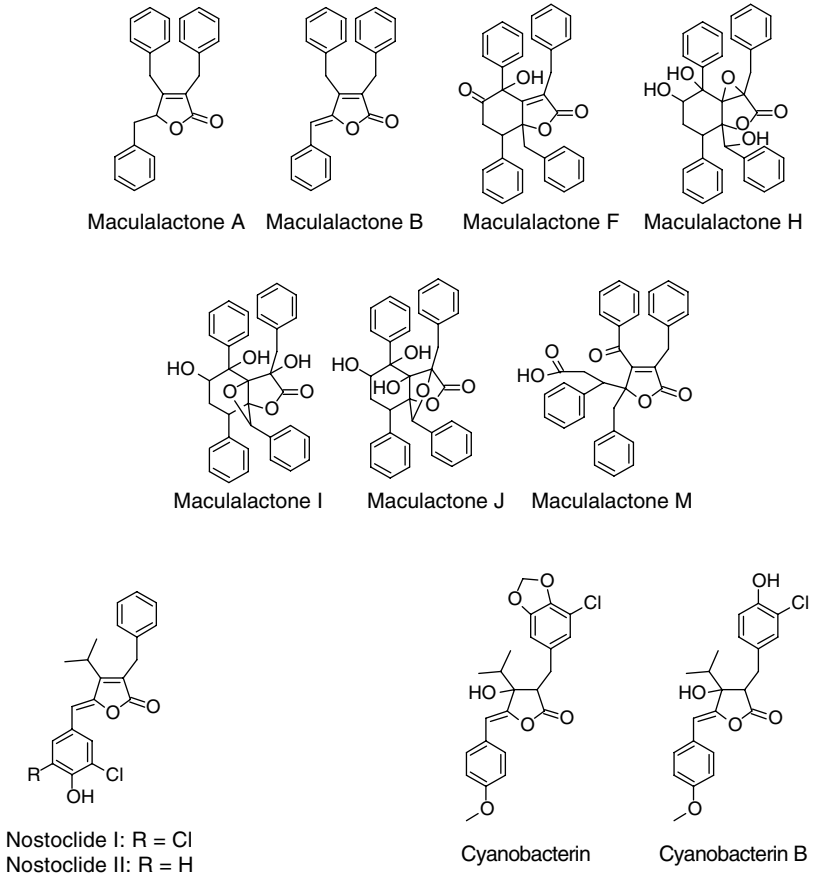


FIG. 50. The maculalactones and related compounds.

C. NONAROMATIC COMPOUNDS

Two small compounds (Fig. 51A) that appear to be cyclized products of a reduced form of citric acid have been reported from *Lyngbya* (Todd and Gerwick, 1995a). One, which appears to derive from condensation with bicarbonate, was the first reported cyclic carbonate from a marine organism. The other is a lactone resulting from cyclization. Another γ-butyrolactone (Fig. 51B) was reported from *Lyngbya* (Ainslie *et al.*, 1986) that had the opposite chirality to an otherwise identical compound resulting from hydrolysis of oscillatoxin A from *Schizothrix* and *Oscillatoria* (Moore *et al.*, 1984a).

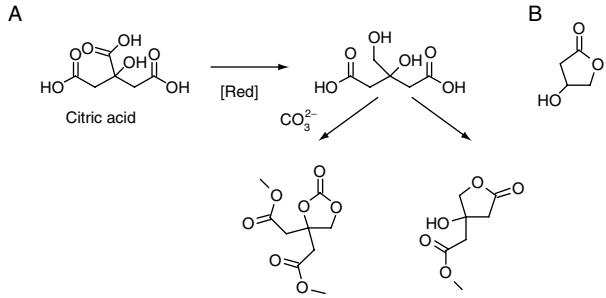


FIG. 51. Various other small molecules from cyanobacteria. (A) A carbonate and lactone that appear to be derived from citrate. (B) A small lactone.

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Pathways to Discovering New Microbial Metabolism for Functional Genomics and Biotechnology

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I. Introduction

The elucidation of fundamental metabolic pathways has been one of the shining achievements of twentieth century science. Every year, thousands of students of biology and chemistry learn the chemical structures and fundamental reactions of metabolism. In their coursework, students learn about the chemical logic underlying how metabolic reactions are linked to provide for the energy and materials of a living cell. Unfortunately, some students also acquire the misconception that metabolism is a static science, a necessary burden of memorization before moving on to the dynamic new vistas of biology such as genomics. This is perhaps accentuated by well-intentioned words in some of the widely used biochemistry textbooks; for example, ''... the chemical basis of many central [biological] processes are now understood'' (Stryer, 1988). Or, for example, in the textbook *Genomics*, it is stated, ''... by the time the human genome is completely sequenced, coding regions will be identifiable with almost perfect accuracy, and most new genes will carry in their sequence immediately recognizable clues about function'' (Cantor and Smith, 1999). These statements

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may lead students to believe that most metabolism, most biological function, is well understood at the fundamental chemical level.

The major thesis of this chapter is that most fundamental reaction-types catalyzed by living systems remain to be uncharacterized. How can we know what is unknown? To do that, one must systematically organize what is known in one domain of science and turn to other domains of science for insight into what is missing. Initially, this chapter describes the organization of metabolic information within the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD). Then, another scientific domain, the natural product chemical literature, has been surveyed to reveal that many of the chemical functional groups produced by biological systems are yet uncharacterized with respect to metabolism. This might at least partly underlie the issue of assigning a biological function to genes in microbial genomes, many of which currently are ascribed to be of ''unknown function'' or ''conserved hypothetical.'' Finally, examples of, and approaches for, new discovery will be discussed.

II. Defining the Hypothesis That Most Metabolic Reactions Are Yet to be Discovered

Metabolic reactions can be categorized based on the type of reaction and the chemical functional group acted upon. This is the general approach taken by the Enzyme Commission (EC) in developing a hierarchal numbering system for enzyme nomenclature. In the EC system, the fundamental reaction type is assigned at the highest level: one for an oxidation–reduction reaction, two for a group transfer reaction, and so on. This has been a very useful scheme for providing a nomenclature for enzymes, which is increasingly needed for tagging enzymes with the rise of databases and other computational tools in biological sciences.

The way our group has chosen to organize metabolism focuses initially on the chemical functional group undergoing a reaction, and the type of reaction it undergoes is secondary. This is more analogous to the approach taken by synthetic organic chemists, who seek to build up or degrade molecules, a process akin to metabolism. In devising a synthetic scheme, one must think first about how to transform a particular functional group into another functional group. Secondarily, thought is then given to the reagent and conditions used to best carry out that transformation with the optimization of yield and selectivity.

The UM-BBD organizes information in this manner (Ellis *et al.*, 2006). There are $\sim$1000 chemical substances in the database, undergoing more

than 1000 unique reactions, but they all fall into one or more clusters of 60 chemical functional groups (Fig. 1). About half of those functional groups are covered in biochemistry textbooks. This includes such groups as esters, amides, amines, alcohols, and thiols. The UM-BBD covers many biochemical functional groups that are considered ''exotic,'' for example the bacterial metabolism of organomercurials, arsenic compounds, and organosilicon compounds.

If one turns to the natural product chemical literature, one can identify over 100 functional groups (Wackett and Hershberger, 2001). Most natural products come from plants and microbes and they are derived from enzyme-catalyzed reactions. However, in most cases, the enzymes and genes involved in these reactions have not been identified. This observation has several consequences.

First, this illustrates that we are ignorant of a significant amount of metabolism that occurs on earth; the reactions, enzymes, and genes are still obscure. Second, the gene sequences that are responsible for these reactions are unknown. This is illustrated by the failure of genomics to identify functions for as much as half of the identified genes in environmental bacteria (Ward and Fraser, 2005). This is compatible with the assertion here that perhaps half of metabolic reaction types are unknown. Identification of the novel metabolic genes will surely result in ascribing functions to at least some of the currently unknown-function genes in the genome-sequencing projects. A third ramification of the hypothesis is that one can discover new biological reactions that can be exploited for biotechnology. Novel microbial reactions have continually provided new sources of biocatalysts for industrial exploitation, of which a few examples are shown in Fig. 2.

III. Organization of Existing Metabolic Information

There are billions of different enzymes if one defines a unique enzyme as having a unique amino acid sequence. There are many isofunctional enzymes differing by 1–80% in sequence identity. Many isozymes show differing substrate specificity but the type of reaction they catalyze is the same. With such a bewildering array of enzymes, how does one define what constitutes a fundamentally different metabolic reaction?

In the UM-BBD, reactions are organized into the type of functional group undergoing transformation (Fig. 1). There are 60 different functional groups described. Each functional group can further undergo a variable number of fundamentally different types of reactions. On average, there are four different reactions per functional group. Figure 3

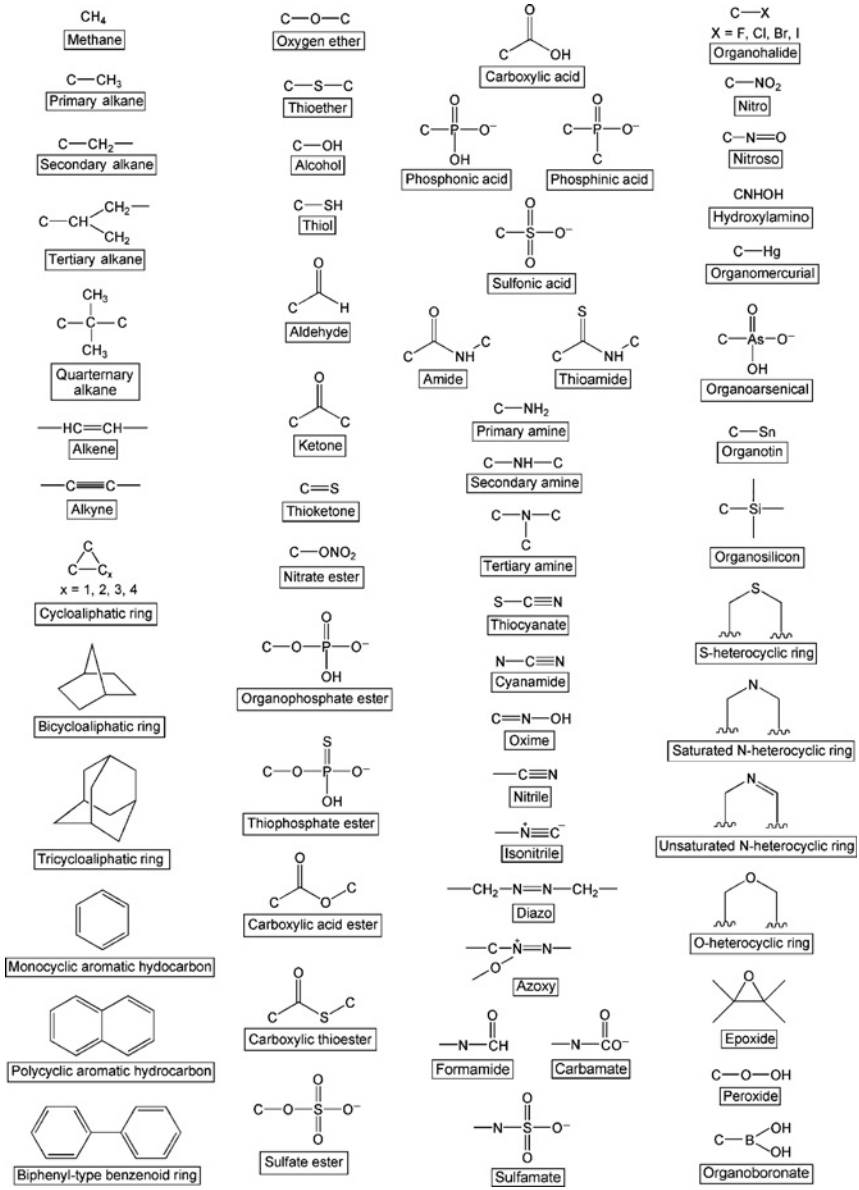

FIG. 1. The 60 organic functional groups that represent functional group metabolism in the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD).

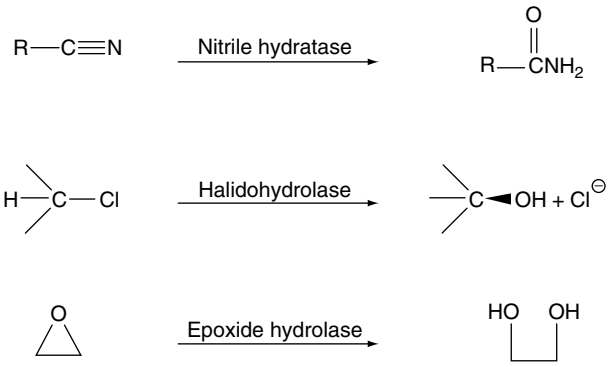

FIG. 2. Recently studied microbial enzymic reactions that have become important in the biotechnology for chemical synthesis.

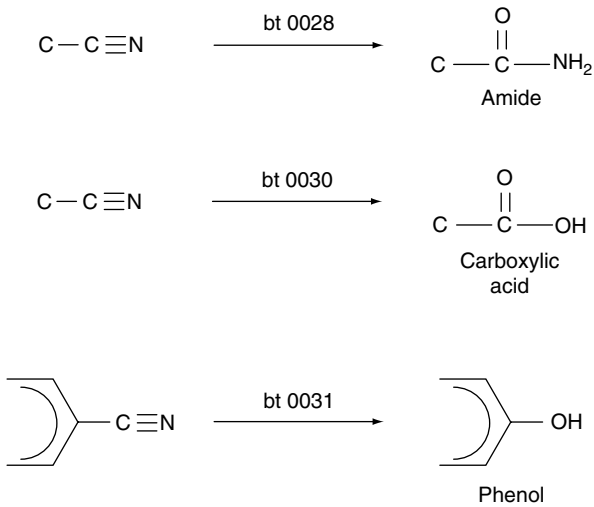

FIG. 3. Three biotransformation (bt) rules describe the general mechanisms by which microorganisms metabolize the nitrile functional group.

shows an example set of three reactions known for the nitrile functional group. Enzymes are known to add water to the carbon–nitrogen triple bond; one atom of water to generate an amide (nitrile hydratase) or two atoms of water to generate a carboxylic acid (nitrilase). There is another reaction known in which an aromatic carbon atom bearing

the nitrile group is hydroxylated with displacement of cyanide. Each reaction type has a separate biotransformation (bt) rule number.

In general, each reaction type is correlated to a metabolic rule in the UM-BBD Pathway Prediction System (PPS) (Hou *et al.*, 2004). There are ~240 metabolic rules in the PPS. The PPS, as the name suggests, is used to predict metabolic pathways for unknown compounds based on known metabolic reaction types. As discussed in Section II, there are also many unknown reaction types. Thus, there is a significant amount of metabolism that cannot be predicted currently based on existing knowledge.

IV. Approaches for New Discovery

Currently, facilities exist to screen proteins generated by genes of unknown function via a series of standardized enzymatic assays (Ahari *et al.*, 2005). The assays are often designed to test for the transformation of esters, amides, amines, alcohols, and other well-studied functional groups. As such, the screens are designed to find proteins of common function with novel sequences. It is much more difficult to use high-throughput screening to find new reactions, since assays for biochemical reactions with previously unstudied functional groups are generally not available.

New reaction types in biology typically are found via dedicated efforts to find a specific enzyme when a novel functional group or reaction is considered particularly interesting or financially rewarding to study. For example, Diels-Alder condensation reactions were previously considered to be in the purview only of organic chemists. In 1993, Gouverneur *et al.* described a catalytic antibody that would catalyze a Diels-Alder reaction with specifically designed compounds (Gouverneur *et al.*, 1993). At that time, the reaction was not thought to occur in biological systems naturally. However, that idea was dispelled as people investigated some important biosynthetic reactions in the production of polyketide antibiotics. In 2003, Ose *et al.* described the X-ray structure for a naturally occurring diels-alderase enzyme (Ose *et al.*, 2003).

The discovery of new biocatalysis is important for fundamental studies in genomics and discovering new biocatalysts for commercial purposes. In our laboratory, a broad-based screening program was initiated to identify new microbes, enzymes, and genes that metabolize biochemically obscure organic functional groups. The screening program focused on catabolic reactions for ease of predicting likely end-products of a given functional group transformation that would yield a

compound which many soil prokaryotes could assimilate to meet their carbon, nitrogen, or energy needs. Since this metabolism might exist in only a small fraction of the microorganisms present in a particular soil, enrichment culture methods were used to amplify the microorganisms possessing the metabolic reaction(s) of interest. Pure cultures of the microorganisms could then be obtained. Each microbial pure culture was subjected to 16S rRNA sequencing to obtain taxonomic information. Of 10 organisms identified, each was capable of metabolizing a different functional group, 6 different genera were obtained. These data suggested that the capacity for novel metabolism is not constrained to a narrow range of taxa. The isolates obtained include *alpha* and *gamma* proteobacteria, low G + C Gram-positive bacteria, high G + C Gram-positive bacteria, and a filamentous fungus. This is somewhat surprising given that the enrichment conditions were uniform except for the growth substrate. This suggests that novel metabolism can be found broadly in the prokaryotic world.

V. Newly Discovered Microbial Metabolism

A. Azetidine Ring-Opening Metabolism

With pure cultures thus obtained, direct evidence of metabolism could be demonstrated using appropriate chromatographic techniques, principally high-pressure liquid chromatography (HPLC) and thin layer chromatography (TLC). The reactions identified would have been difficult to predict from known metabolic reactions. For example, metabolism of the plant natural product L-azetidine-2-carboxylic acid (ACA) was studied using isolated *Pseudomonas* strain A2C. ACA is structurally analogous to the amino acid L-proline. In fact, ACA is synthesized by plants as a toxin that mimics proline (Fowden, 1956). Susceptible organisms take up ACA and incorporate it into proteins (Fowden and Richmond, 1963). Because ACA is composed of a four-membered ring compared to a five-membered ring for proline, the bond angles are different for each compound when incorporated into proteins. Since proteins have evolved for the specific bond angles achieved with proline, some proteins become completely inactive when incorporating ACA, with lethal effects on the organism.

Thus, one might have predicted that ACA would be catabolized via biochemical mechanisms analogous to those known for proline. Proline is catabolized via oxidation of the cyclic amine to an imine and hydrolytic opening of the imine (Scarpulla and Soffer, 1978). However, ACA is metabolized differently. *Pseudomonas* sp. A2C has an enzyme that

uses the inherent angle strain in the four-membered ring to directly hydrolyze the cyclic amine's carbon–nitrogen bond, opening the ring to produce 2-hydroxy-4-aminobutyrate that is nontoxic (Dunnill and Fowden, 1965). 2-Hydroxy-4-aminobutyrate has been reported to undergo transamination to capture the amino group into cellular metabolism. Thus, the azetidine ring-opening reaction detoxifies and sets up the compound for nitrogen capture at the same time. The enzyme encoding AC hydrolytic opening was found to be a homologue of 2-haloacid dehalogenases (C. Gross, unpublished data). This represents a very large gene family in GenBank. However, a large majority of the genes have been identified by computer annotation only during the many microbial genome projects conducted over the last several years. As such, most have never been tested for dehalogenase activity nor has it been questioned whether this dehalogenase activity would have any meaningful context in the overall metabolism of the organism. There are at least several bacteria that contain homologues to AC hydrolase that may in fact be active in the hydrolytic ring opening of azetidine-2-carboxylate.

B. Arylboronic Acid Metabolism

In another set of studies, an *Arthrobacter* sp. capable of growth on phenylboronic acid was used to determine the enzymological and genetic basis of the metabolism (see Fig. 4). The bacterial metabolism of organoboron compounds has been little studied, although boron is required for proper biological function in microbes and plants. Boron-containing salts have long been included in bacterial growth media (Stanier *et al.*, 1966). Organoboron natural products are known, for example the antibiotics boromycin produced by *Streptomyces* sp. (Dunitz *et al.*, 1971) and the tartrolons produced by *Sorangium cellulosum* (Irschik *et al.*, 1995). Phenylboronic acid was used as an enrichment substrate to obtain bacteria capable of growing on phenylboronic acid as their sole carbon source. This necessitated that one or more aromatic ring carbon atoms be assimilated with likely carbon–boron bond cleavage. A pure culture was obtained and it was determined to be an *Arthrobacter* sp. and denoted as strain PBA. Metabolic studies with the *Arthrobacter* sp. strain PBA demonstrated that phenol was an intermediate in phenylboronic acid metabolism (Negrete-Raymond *et al.*, 2003). Experiments were conducted using a stable heavy isotope of oxygen in an atmosphere containing $[^{18}O]$-O_2 or, in separate experiments, in water containing $[^{18}O]$-H_2O. Phenol was isolated in each of those experiments and tested for $[^{18}O]$-incorporation by mass spectrometry. The $[^{18}O]$-label was only incorporated following incubations with

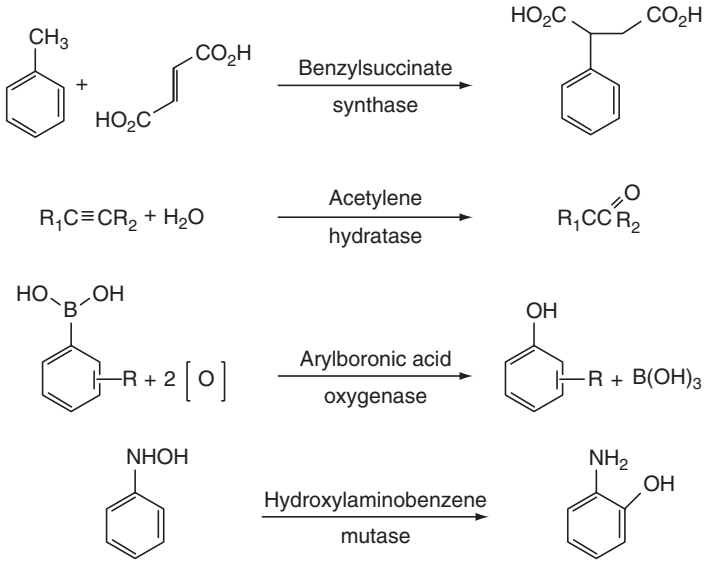

FIG. 4. Four recently discovered microbial enzyme-catalyzed reactions that are broadly functional with a given organic functional group.

phenylboronic acid under an [^{18}O]-O_2 atmosphere. These data indicated that the oxygen in the product was derived via an oxygenase-catalyzed reaction. A range of arylboronic acids were metabolized suggesting that the mechanism was generalizable to other boron-containing compounds. These biological experiments are reminiscent of organochemical studies in which phenylboronic acids have been shown to react with hydrogen peroxide to generate phenylborate esters with incorporation of oxygen from hydrogen peroxide into the ester (March, 1997). Phenylboronic acid esters hydrolyze in water by attack on the boron center with phenol as the leaving group, consistent with the type of mechanism inferred from the oxygen incorporation experiments described here.

C. THIOAMIDE METABOLISM

Thioamides, unlike amides, are rare in biological systems. As a result, amide metabolism is well known but comparatively little has been reported on thioamide metabolism. Thioamide natural products are known, for example the copper-chelating compound methanobactin produced by *Methylosinus trichosporium* OB3b (Kim *et al.*, 2004). Intuitively, it might be predicted that thioamides are metabolized via

hydrolytic cleavage of the carbon–nitrogen bond, reminiscent of amides. This prediction is consistent with studies showing that thioamides serve as the sole nitrogen source supporting the growth of microbial enrichment cultures (Hou *et al.*, 2004). However, the mechanism of thioamide metabolism was not established in that study. More recently, a pure culture of *Ralstonia pickettii* strain was isolated on thioacetamide as the sole nitrogen source (Dodge *et al.*, 2006). *R. pickettii* also metabolized thiobenzamide with the accumulation of benzamide and benzonitrile. Thioamide *S*-oxides were shown to be intermediates in metabolism. Thioamides were established to be oxidized to thioamide *S*-oxide and a presumptive second oxygenation to yield an *S*-dioxo intermediate. The latter intermediate is unstable and would be expected to eliminate spontaneously producing either a nitrile or an amide, or a mixture of both. This mechanism fits the observed products. Again, this type of assimilation mechanism could not be predicted without isolating bacteria and conducting experimental studies. These data, in total, argue for continuing efforts for the discovery and elucidation of novel microbial metabolism.

D. Organobismuth Metabolism

Bismuth subsalicylate, the active ingredient in Pepto-Bismol, was used as the carbon source in enrichment cultures. Bismuth subsalicylate has been used to treat ulcers and other gastrointestinal disorders for several centuries (Sadler *et al.*, 1999). Despite this prolonged medical usage, the mechanism by which bismuth subsalicylate inhibits gastrointestinal microbes is not understood in detail. Toxicity is dependent on bismuth(III). Thus, the organism isolated in our enrichment cultures must be able to assimilate salicylate and be resistant to bismuth(III).

Bismuth resistance genes in microbes have been identified on plasmids in *Staphylococcus aureus* and found to be linked to cadmium/zinc and mercury resistance genes (Novick and Roth, 1968). Bacteria exposed to bismuth have been shown to accumulate bismuth but the molecular details of this sequestration are largely unknown. In anaerobic environments, trimethylbismuth gas effluent has been detected and attributed to microbial metabolism (Bentley and Chasteen, 2002).

In our studies, a filamentous fungus, identified as a *Fusarium* sp., was isolated with bismuth subsalicylate as the sole carbon source (Dodge and Wackett, 2005). *Fusarium* sp. BI accumulated bismuth intracellularly during growth on bismuth subsalicylate and the accumulated bismuth was found to be sequestered in polyphosphate granules, concentrated

in phosphorus-rich inclusions in specimens prepared for electron microscopy. This might underlie the organisms ability to be resistant to bismuth(III). *Fusarium* sp. BI metabolized salicylate via transformation to catechol and ring cleavage via catechol 1,2-dioxygenase.

VI. Significance of New Discoveries in Novel Functional Group Metabolism

Studies on the microbial biodegradation of nitrobenzene compounds have yielded new knowledge of functional group biochemistry. The metabolic pathway was observed to proceed via reduction of the nitro group to a nitrosobenzene and further reduction to a hydroxylamine (Somerville *et al.*, 1995). Surprisingly, hydrozylaminobenze was found to undergo a biological equivalent of the organic-named reaction known as the Bamberger rearrangement (Nishino and Spain, 1993). The enzyme catalyzing this novel reaction has become known as hydroxylaminobenzene mutase (He *et al.*, 2000). The mutase gene *habA* from *Pseudomonas pseudoalcaligenes* JS45 was sequenced and GenBank was searched for matches (Davis *et al.*, 2000). One of the homologues to *habA* is hypothetical protein Rv3078 that was identified in the genome-sequencing project for *Mycobacterium tuberculosis*, the causative agent of the widespread lung disease tuberculosis. On the basis of new knowledge of what reaction HabA might catalyze, hypothetical protein Rv3078 is proposed to underlie the resistance to nitroaromatic therapeutic agents, an emerging class of last-line-of-defense antibiotics for tuberculosis infections (Di Santo *et al.*, 1998; Murugasu-Oei and Dick, 2000; Stover *et al.*, 2000). Thus, the discovery of new functional group metabolism, in this example, may contribute key insights into an infectious disease-resistance mechanism with the potential for developing therapies to overcome resistance to current antitubercular drugs.

VII. Use of Recently Discovered Biocatalysis Industrially

In addition to fundamental advancements in genomics, novel reactions can be incorporated into the toolkit of enzyme-based industrial chemistry. In the past, enzymes using water as a cosubstrate have dominated in industrial processes due to their ease of handling and relatively greater stability under extreme conditions (Panke *et al.*, 2004). Increasingly, there is interest in expanding this enzymic repertoire and this will be a key feature of a broad-based usage of biologically based renewable resources by the chemical industry.

An intriguing enzyme in this regard is the class of enzymes that catalyze carbon–carbon bond formation while introducing a chiral center during the anaerobic metabolism of hydrocarbons (Fig. 4). This is now known to be a widespread mechanism for the anaerobic catabolism of linear alkanes, branched alkanes, and alkylaryl compounds (Spormann and Widdel, 2000). The most well-studied example of this mechanism is the reaction catalyzed by the enzyme benzylsuccinate synthase, found in anaerobic bacteria that grow on toluene (Achong *et al.*, 2001). The enzyme has been purified and shown to contain a stable organic radical using EPR (Krieger *et al.*, 2001). The radical signal was observed to correlate with the level of activity. The reaction is proposed to occur via a radical addition of the toluene benzyl radical to fumaric acid. Despite the radical intermediate, the reaction occurs with complete enantiocontrol, a remarkable reaction that could not readily be duplicated by chemical reagents. Moreover, the reaction occurs with such a wide range of compounds that it could be a route to the synthesis of thousands of chiral molecules. This is an example of the potential for new biochemical reactions to be exploited for biotechnological gain.

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Biocatalysis by Dehalogenating Enzymes

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I. Introduction

Dehalogenases catalyze the cleave of carbon–halogen bonds, a reaction that is of key importance for the microbial utilization of halogenated organic compounds as carbon source, electron donor, or electron acceptor. The first reports on dehalogenases concern enzymes involved in chloroacetate and fluoroacetate metabolism (Davies and Evans, 1962; Goldman, 1965; Jensen, 1960). A few years later, Castro and Bartnicki (1968) described an enzyme activity that converted 2,3-dibromo-1-propanol to epibromohydrin with bromide release. These pioneering studies were followed by many papers in which new dehalogenase reactions were described and the results have been reviewed several times (de Jong and Dijkstra, 2003; Fetzner and Lingens, 1994; Janssen, 2004; Janssen *et al.*, 1994). Many dehalogenases were purified and characterized and genes were cloned and sequenced. More than a dozen different types of dehalogenating enzymes were identified, including coenzyme-dependent enzymes, redox proteins, and hydrolytic enzymes. Dehalogenases appear with very different structural folds, reaction types, and catalytic mechanisms. For example, several reductive

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dehalogenases that replace a chlorine substituent by a hydrogen possess a corrinoid cofactor typical for an electron transfer mechanism, whereas haloalkane dehalogenases belong to the α/β-hydrolase fold family, a group of proteins of which most members catalyze hydrolytic reactions via a covalent alkyl-enzyme intermediate, reminiscent to the acyl-enzyme intermediate of classical serine proteases.

Studies on dehalogenases have traditionally been motivated by the environmental relevance of their substrates. For example, reductive dehalogenases are responsible for the anaerobic dechlorination of notorious environmental pollutants like trichloroethylene or chlorobiphenyls, whereas certain glutathione-dependent dehalogenases detoxify the frequent groundwater pollutant dichloromethane. There is also a long-standing interest in using dehalogenases for industrial biocatalysis. In fact, polluted environments where microorganisms occur that have adapted to mineralize or transform synthetic compounds may be a rich source for a diversity of biocatalysts which can perform transformations that are potentially useful for the manufacture of fine chemicals. Biocatalytic applications (Swanson, 1999) that were explored already years ago are based on the enantioselectivity of some 2-chloropropionic acid dehalogenases (Taylor, 1997) and the use of halohydrin dehalogenase (then termed halohydrin epoxidase) for converting halohydrins to epoxides (Geigert *et al.*, 1983; Kasai *et al.*, 1998). More recently, dehalogenases have been further explored for enantioselective conversion of other halocarboxylic acids and their esters and haloalkanes, as well as for their applicability in recycling and detoxifying trichloropropane. Nonnatural reactions have also been investigated, employing different nucleophiles in reverse dehalogenation reactions, which is important for the synthesis of the important pharmaceutical intermediate ethyl 4-cyano-3-hydroxybutyrate.

In this chapter, three types of dehalogenase will be discussed: halocarboxylic acid dehalogenases, haloalkane dehalogenases, and halohydrin dehalogenases. Emphasis will be on the microbial origin and distribution of these enzymes, their biochemical properties, and their engineering and use in biocatalysis.

II. Halocarboxylic Acid Dehalogenases

Microorganisms that degrade 2-chloropropionic acid or chloroacetic acid are easily enriched from soil samples, and they produce haloacid dehalogenases that cleave a carbon–halogen bond in a conversion that is a net hydrolysis (Fig. 1A). The halocarboxylic acid dehalogenases

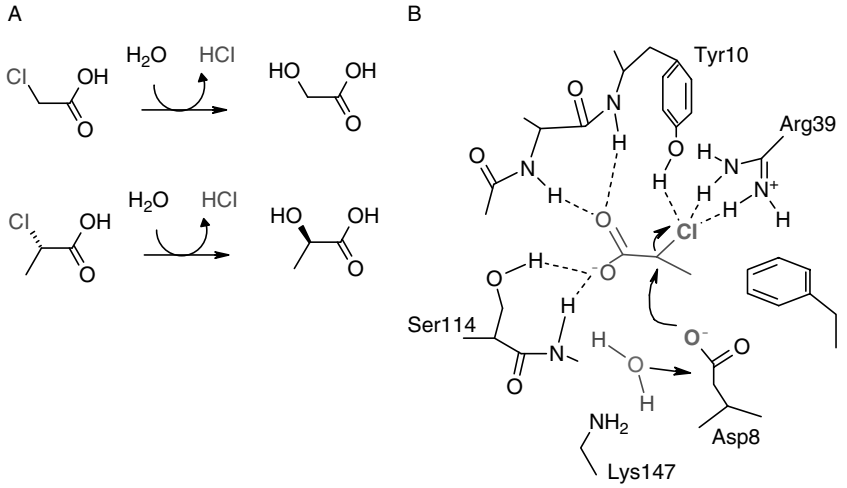

FIG. 1. (A) Reactions catalyzed by DhlB, a type II haloacid dehalogenase of the HAD superfamily of proteins that is selective for (S)-2-chloropropionic acid. (B) Active site groups, with residues involved in catalysis indicated with the number they have in the DhlB sequence. Asp8 is the nucleophile. Its position is conserved within the HAD superfamily.

can be divided in at least three different phylogenetic groups, all of them converting the substrate with inversion of configuration at the substituted carbon atom (Hill *et al.*, 1999). The group I enzymes, of which the D-2-haloacid dehalogenase from *Pseudomonas putida* AJ1 (UniProtKB/Swiss-Prot entry Q52086) and the DL-2-haloacid dehalogenase from *Pseudomonas* sp. 113 (O06652) are the best studied examples, are mostly selective for (*R*)-(+)-2-haloalkanoic acids or nonselective (Smith *et al.*, 1990). The dehalogenation reaction likely proceeds without formation of a covalent substrate–enzyme intermediate, as suggested by ^{18}O incorporation experiments. Instead, it was proposed that a water molecule directly displaces the halogen (Nardi-Dei *et al.*, 1999). No structure is available, so details about the catalytic mechanism are still lacking, although five residues have been identified by mutagenesis that are essential for catalysis (Nardi-Dei *et al.*, 1997). Sequence analysis does not provide clear information about these enzymes since there is also no clear homology with enzymes of which the structure and mechanism have been solved.

The group II haloacid dehalogenases have been better characterized with structures available for the enzymes from *Pseudomonas* sp.

YL (Q53464; Hisano *et al.*, 1996; Li *et al.*, 1998) and *Xanthobacter autotrophicus* GJ10 (Q60099; Ridder *et al.*, 1997, 1999). These proteins have a two-domain structure with the active site located between them. The conserved overall topology of a number of phosphatases, phosphoryl transferases, and the group II haloacid dehalogenases has led to the identification of the so-called HAD superfamily of proteins (Koonin and Tatusov, 1994). Members can cleave carbon–chlorine, carbon–phosphorous, or phosphorous–oxygen bonds. The group II haloacid dehalogenases are more widespread than the group I enzymes as indicated by analysis of genome sequences. They are readily identified in sequenced microbial genomes. So far, members of the group II dehalogenases are active with the L-(*S*)-2-chloropropionic acid but not with the (R)-enantiomer of 2-chloropropionic acid. Catalysis proceeds via a covalent substrate–enzyme intermediate that is formed by nucleophilic attack of an aspartate oxygen on the Cα carbon of the substrate, leading to nucleophilic displacement of the halide. A halide-binding site, formed in part by an arginine, stabilizes the leaving group (Fig. 2B).

A third family of haloacid dehalogenases is formed by fluoroacetate dehalogenases. Like haloalkane dehalogenases, these belong to the α/β-hydrolase fold superfamily of enzymes and act via covalent catalysis. A structure of the enzyme from *Burkholderia* sp. FA1 (Kurihara *et al.*, 2003) has been deposited (BAE94252, PDB code 1Y37_B) but details have not yet been published.

Older literature mentions the existence of dehalogenases that retain the stereochemical configuration at the carbon atom that carries the halogen (Weightman *et al.*, 1982). None of these reports have been substantiated by detailed molecular analysis of the enzymes involved.

Biocatalytic application of haloacid dehalogenases has been realized. The (*R*)-selective (group I) enzyme from *P. putida* AJ1 is used in the form of an engineered whole-cell biocatalyst that overproduces the enzyme and lacks the dehalogenase with opposite enantioselectivity for the kinetic resolution of (*R,S*)-2-chloropropionic acid. This process for the production of L-(*S*)-2-chloropropionic acid was developed by ICI (later Zeneca and now Avecia), replacing an older technology that was based on the chlorination of fermentatively produced optically active lactic acid (Taylor, 1997). The product can be used for the manufacture of phenoxy herbicides, such as mecaprop (2-methyl-4-chloro-2-phenoxypropionic acid), which are plant auxin analogues and act as growth disruptors. The use of the enantiopure active form reduces environmental burden of herbicide use. Immobilization of haloacid dehalogenases with different methods has been studied with emphasis on carrier behavior and enzyme stability (Diez *et al.*, 1996; Ordaz *et al.*, 2000;

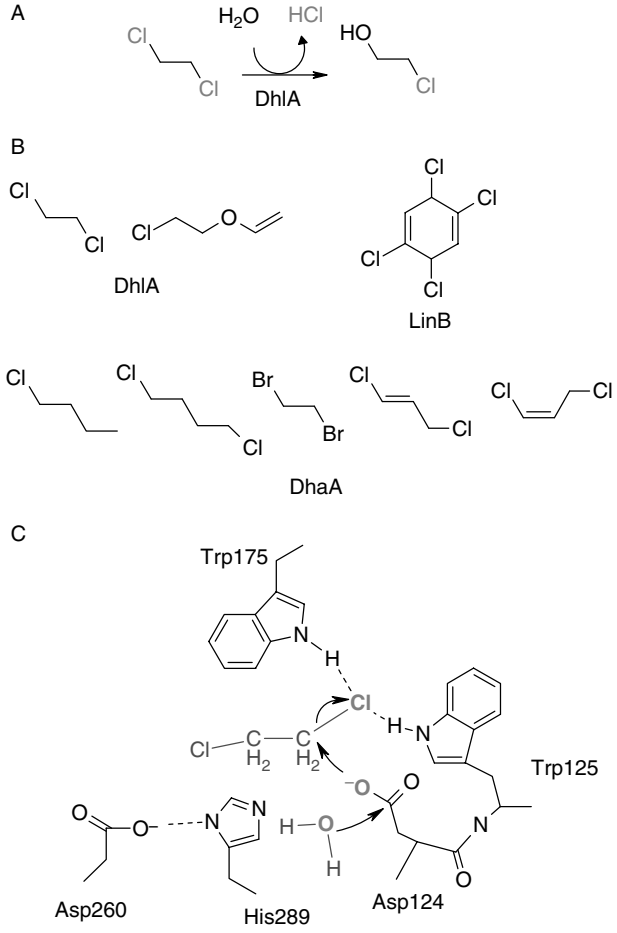

FIG. 2. Haloalkane dehalogenase substrates. (A) Hydrolysis of 1,2-dichloroethane, a typical substrate for a DhlA-type haloalkane dehalogenase. (B) Some compounds converted by haloalkane dehalogenase producing organisms. (C) Mechanism of DhlA, a haloalkane dehalogenase of the α,β-hydrolase fold superfamily. In DhlA, Trp125 and Trp175 are involved in halide binding; in DhaA and LinB, the role of Trp175 is taken over by an asparagine (Asn38 in LinB or Asn41 in DhaA).

Parker and Colby, 1995). The best results were obtained with controlled pore glass that was activated with diazonium salt for covalent enzyme coupling which was done in the presence of substrate (Parker and Colby, 1995).

III. Haloalkane Dehalogenases

A. Properties, Occurrence, and Mechanisms

Haloalkane dehalogenases were first characterized from organisms such as *X. autotrophicus* growing on 1,2-dichloroethane (UniProtKB/Swiss-Prot entry P22643) and *Rhodococcus rhodochrous* growing on 1-chlorobutane (P59336). The X-ray structures of three members of this enzyme family have been characterized, and it appeared that they belong to the α/β-hydrolase fold superfamily of proteins, which encompasses mostly hydrolytic enzymes such as lipases and esterases. Catalysis proceeds via a covalent intermediate that is formed by attack of an aspartate present in a DWG sequence motif on the carbon atom to which the displaced halogen is bound (Fig 2). The covalent intermediate is hydrolyzed by water, and a distinct halide-binding site is present (Janssen, 2004; Verschueren *et al.*, 1993). The active site is located on top of the main domain, and it is covered by a variable cap domain that creates a rather occluded cavity. There is also a distinct halide-binding site. The substrate spectrum of these enzymes varies. The haloalkane dehalogenase from *X. autotrophicus* has a rather narrow substrate range, while that of the LinB enzyme from *Sphingobium japonicum* (LinB, entry P51698) that is involved in the dehalogenation of the lindane intermediate tetrachlorocyclohexadiene is much broader.

Haloalkane dehalogenases are rather widespread. Their genes are abundantly present in various environmental or pathogenic bacteria of which the genome has been sequenced and haloalkane dehalogenase sequences are also easily recovered from the Sargasso sea database (Janssen *et al.*, 2005). For example, *Mycobacterium tuberculosis* has at least three haloalkane dehalogenases (Jesenska *et al.*, 2005). In agreement with this notion, organisms that produce a haloalkane dehalogenase are relatively easily isolated from soil samples. In our hands, the most frequently found dehalogenases are the DhaA-type enzyme detected in bacteria that grow on chlorobutane (Poelarends *et al.*, 2000; similar to AAC15838) and related compounds and the DhlA-type enzyme (P22643) that has repeatedly been detected in organisms that grow on 1,2-dichloroethane. It seems that enrichment and genomic approaches scan very different galaxies of sequence space because very little of the diversity detected in genome sequences has been recovered in enrichment cultures and vice versa.

Research on the use of haloalkane dehalogenases in biocatalysis has focused on the biotransformation of specific problem compounds, most notably 1,2,3-trichloropropane, and the possibility to use the enzyme in enantioselective transformations.

B. Enantioselectivity

The enantioselectivity of haloalkane dehalogenases was investigated by Pieters *et al.* (2001) and by Prokop *et al.* (2006). The enantioselectivities observed by Pieters *et al.* were rather low, but Prokop *et al.* found higher E values, especially with the haloalkane dehalogenase from *Bradyrhizobium japonicum* (P59337) which displaced a high enantioselectivity with 2-bromopentane ($E > 100$). An E value of 50–100 is often considered the lower limit for developing an industrially attractive conversion. The large natural diversity of haloalkane dehalogenases that is predicted to exist by genomic information suggests that even more attractive enzymes may be found.

C. Trichloropropane Conversion

Work on 1,2,3-trichloropropane dehalogenation has been motivated by its presence in waste from epichlorohydrin manufacture, which has caused groundwater contamination in the past and still necessitates high-cost processes for the treatment of waste streams generated at production sites. A broad study to develop a biocatalytic process for trichloropropane removal was carried out in collaboration between Dow Chemical and Diversa Corporation. Work focused on improving the performance of a known haloalkane dehalogenase (DhaA, variant P59336) by directed evolution, mainly aiming at enhanced stability and activity (Gray *et al.*, 2001), as well as on the isolation of new dehalogenases from environmental gene libraries (Gray *et al.*, 2003) and the development of suitable bioreactors (Dravis *et al.*, 2001). Our laboratory has also investigated TCP degradation, exploring monooxygenase-mediated conversion and engineering of a DhaA-type haloalkane dehalogenase.

Gene site-saturation mutagenesis of DhaA was used by Gray *et al.* (2001) to create all possible single site mutants, which was followed by high-throughput screening for activity and temperature stability. More stable variants were indeed found. By combining the single mutations, a haloalkane dehalogenase mutated at five positions and an eightfold mutated variant were obtained. Whereas the wild type had a half-life of only 11 min at 55°C, the fivefold mutant had a half-life of 20 days, and the eight mutations variant had a half-life even of 230 days at this temperature. Furthermore, the wild type was irreversibly inactivated with an apparent melting temperature of 65°C, while the eightfold mutant showed a T_m of 73°C and fully renatured after heat-induced unfolding. Although the intrinsic kinetic properties of the enzyme toward trichloropropane were not much improved (1.4-fold), the 8-fold mutant enzyme could be used at

higher temperature. The improved performance of the mutants was also evident when the eightfold variant was immobilized on cross-linked alumina and used for continuous removal of trichloropropane. The productivity, defined as the initial rate of substrate conversion per amount of enzyme per hour, increased 1.4-fold at 55°C. Over a longer period, the performance of the engineered dehalogenase was 25-fold better (in terms of grams of substrate degraded per gram of enzyme during 4 hour) because of its improved stability.

Studies in our laboratory were aimed at enhancing the activity toward 1,2,3-trichloropropane by directed evolution (Bosma *et al.*, 2002). After error-prone PCR and gene shuffling, a mutant was obtained in which two substitutions caused an increase in selectivity constant (k_{cat}/K_m) of about tenfold. The Cys176Tyr and Tyr273Phe substitutions are close to the active site, and molecular dynamics simulations suggest that the mobility of the substrate in the active site is changed in favor of a more frequent occurrence of a reactive conformation (Bosma *et al.*, 2002). The improved dehalogenase was cloned in a 2,3-dichloro-1-propanol utilizing host, and the resulting recombinant could slowly grow on trichloropropane. Although these improved variants were obtained, a full-scale process for removal or recycling of TCP from groundwater or waste streams has not been implemented due to competitive chemical processes and the modest activity of the dehalogenase, which was improved but so far remained too low to be economically attractive in an application.

Various other approaches have been aimed at enhancing or modifying the activity of haloalkane dehalogenases, for example applying structural analysis (Chaloupkova *et al.*, 2003), ITCHY-like mutagenesis methods (Pikkemaat and Janssen, 2002), or selection of spontaneous mutants (Pries *et al.*, 1994; papers reviewed in Janssen, 2004). The results, which targeted mainly compounds of environmental relevance, gave a wealth of information about dehalogenase selectivity and evolution, but have not yet yielded mutant enzymes that are sufficiently active to be used in a biocatalytic process for industrial synthesis or waste treatment other than for 1,2-dichloroethane removal, the natural substrate of DhlA.

IV. Halohydrin Dehalogenases

A. Isolation, Properties, and Mechanism

The type of reaction catalyzed by halohydrin dehalogenases is different from that of the hydrolytic haloalkane and haloacid dehalogenases (de Jong and Dijkstra, 2003). Activity only is observed with vicinal

halohydrins, and the products of the dehalogenation reaction are an epoxide, a halide ion, and a proton (Fig. 3A). The conversion is reversible, depending on the nature of the leaving group. In the reverse (epoxide-ring opening) reaction, various alternative negatively charged nucleophiles can be accepted such as cyanide and azide. Insight in the biochemistry and catalytic mechanism was obtained when sequences and an X-ray structure became available (de Jong *et al.*, 2003, 2006; Tang *et al.*, 2003; van Hylckama Vlieg *et al.*, 2001). The halohydrin dehalogenases appeared to belong to the short-chain dehydrogenase–reductase (SDR) superfamily of proteins, which are oxidoreductases that use NAD or NADP as the cofactor. However, the halohydrin dehalogenase reaction is not a redox reaction and in agreement with this, the dehalogenases do not have the typical Rossmann fold sequence motif that is conserved in the SDR enzymes, and they do not bind NAD(P). The catalytic residues that are involved in proton abstraction during alcohol oxidation are conserved in the dehalogenases, however, which led to a mechanism for HheC as is shown in Fig. 3B.

Halohydrin dehalogenases are rare enzymes. Unlike haloacid dehalogenases and haloalkane dehalogenases, they do not abundantly appear in the sequenced microbial genomes or in environmental DNA sequences, and thus are not likely picked up in modern screening methods for new biocatalysts employing environmental gene libraries (Janssen *et al.*, 2005). Nevertheless, organisms that degrade chloropropanols and produce halohydrin dehalogenases are relatively easy to isolate after appropriate enrichment, and the enzymes are often well expressed in *Escherichia coli*.

B. Biocatalytic Processes

Halohydrin dehalogenases were explored for their use in biocatalysis already in the 1980s, mainly by Japanese groups, and full-scale whole-cell processes based on their activity have been developed by Daiso Co. This has been described in interesting reviews (Kasai and Suzuki, 2003; Kasai *et al.*, 1998). Briefly, (*R*)-2,3-dichloro-1-propanol is produced by stereoselective degradation of an (*R*,*S*) racemic mixture by *Alcaligenes* sp. DS-K-S38, and (*S*)-2,3-dichloro-1-propanol is obtained with the *R*-selective degrader *Pseudomonas* OS-K-20. The remaining enantiopure dichloropropanols obtained in these kinetic resolution processes are easily converted to enantiopure epichlorohydrin with base. Similarly, (*S*)-3-chloro-1,2-propanediol is produced from a racemic mixture using *R*-selective degradation by *Alcaligenes* sp. DS-S-7G, and the (*S*) enantiomer is obtained when the starting compound is treated with

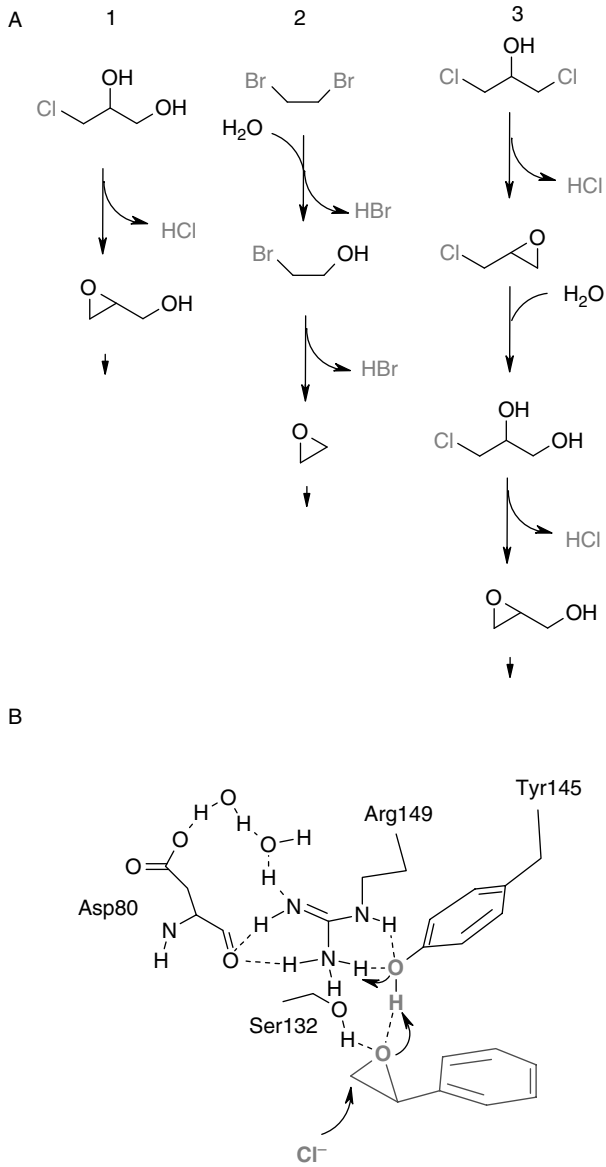

FIG. 3. (A) Catabolic pathways in organisms from which halohydrin dehalogenase-producing bacteria have been obtained. 1, *Arthrobacter* AD2 (HheA) (van Hylckama Vlieg *et al.*, 2001); 2, *Mycobacterium* GP1 (HheB) (Poelarends *et al.*, 1999); 3, *Agrobacterium* AD1 (HheC), *Agrobacterium* NHG3 (Assis *et al.*, 1998), *Corynebacterium* N-1074 (HheA, HheB) (Nakamura *et al.*, 1992). (B) Active site in HheC, showing nucleophilic attack during epoxide ring opening and protonation of the epoxide oxygen by a conserved tyrosine. The halide-binding site is not indicated.

Pseudomonas sp. DS-K-2D1. The remaining 3-chloropropanediols can be converted to the enantiopure glycidols. All these dehalogenation reactions proceed with retention of configuration at the carbon atom carrying the hydroxyl group.

The enantioselectivity of halohydrin dehalogenases can also be used for preparing enantiopure halohydrins by kinetic resolution without complete degradation of the epoxide (Assis *et al.*, 1998). Dependent on the leaving group, or nucleophile if we consider epoxide ring-opening reactions, the equilibrium between epoxide and haloalcohol may be in the direction of the ring-closed product or the ring-opened product. In the case of a halogen substituent the ring-closure reaction will not go to completion, which reduces enantiopurity of the remaining halohydrin in a kinetic resolution. To overcome this problem, a tandem reaction that uses epoxide hydrolase to shift the equilibrium was explored (Lutje Spelberg *et al.*, 1999).

The remarkable enantioselectivity of HheC was investigated by X-ray crystallography and computational work (de Jong *et al.*, 2005). By analyzing crystal structures of enzyme complexed with (*S*)- or (*R*)-*para*-nitrostyrene oxide, it appeared that the nonpreferred (*S*)-enantiomer is bound in an inverted manner, in which the epoxide oxygen is in the position of the terminal carbon atom on which the nucleophilic attack during a normal epoxide ring-opening reaction takes place, and the terminal carbon atom occupies the position of the epoxide oxygen. In this way, the nonpreferred enantiomer can bind in the active site in a similar way as the opposing-preferred enantiomer, that is, without changing the position that a large substituent on the oxirane ring occupies, in this case the *p*-nitrophenyl group. Binding is weaker, however, and obviously a reaction also cannot occur this way.

C. The use of Alternative Nucleophiles

The studies by Nakamura *et al.* (1991, 1994) indicated the possibility to use halohydrin dehalogenase in a pseudotranshalogenation reaction in which the halogen is replaced by cyanide. The conversion of butene oxide to 2-hydroxyvaleronitrile was catalyzed by halohydrin lyase A cloned in *E. coli* from *Corynebacterium* sp. N-1074 (BAA14361). The same enzyme converted 1,2-epoxypropane to β-hydroxybutyronitrile and epichlorohydrin to γ-chloro-β-hydroxybutyronitrile. This indicated that halohydrin dehalogenase may accept alternative nucleophiles in ring-opening reactions.

The use of cyanide in epoxide ring-opening reactions with diverse epoxides has been further explored by Majerić-Elenkov *et al.* (2006a).

It appeared that of the three enzymes that were investigated, HheC (AAK92099) had the nicest enantioselectivity, especially with 2,2-disubstituted epoxides such as 1,2-epoxy-2-methylbutene-3 or 1,2-epoxy-2-methylbutane. Two other enzymes, HheA from *Arthrobacter* AD2 and HheB from *Mycobacterium* GP1, converted the substrates with lower and opposite enantioselectivity. These cyanation reactions of HheC result in carbon–carbon bond formation. In this sense, the halohydrin dehalogenase reaction complements that of hydroxynitrilases that catalyze cyanation of aldehydes and ketones. The hydroxynitrilases produce α-hydroxynitriles, whereas the products of halohydrin dehalogenases are β-hydroxynitriles.

Other nucleophiles that were found to be accepted by halohydrin dehalogenase are azide (Lutje Spelberg *et al.*, 2001) and nitrite (Hasnaoui *et al.*, 2005). With azide, the reaction rates are usually somewhat higher than with cyanide. Enzymatic azidolysis of epoxides proceeded with complete selectivity for the terminal carbon atom of the epoxide ring, unlike the chemical reaction, which favors attack on the secondary carbon atom. The intrinsic enantioselectivity of the enzyme is very high, but the enantiomeric excess (ee) of the product is somewhat reduced due to the occurrence of a chemical side reaction, which also reduces the yield of the remaining enantiomer in a kinetic resolution. Careful optimization of process conditions may improve this, for example through slow dosing of the azide, suppressing chemical attack. Also in azidolysis of styrene oxides, the enantioselectivity was highest with HheC.

In particular cases, the combined use of epoxide ring opening and closure catalyzed by halohydrin dehalogenase can be used for a dynamic kinetic resolution (Lutje Spelberg *et al.*, 2004). The dehalogenase catalyzes a racemization of epibromohydrin in the presence of low concentrations of bromide and an enantioselective azidolytic ring opening of the same compound. The net reaction is a dynamic kinetic resolution that yields azidobromopropanol.

Nitrite is an ambident nucleophile that can attack an oxygen by its nitrogen or oxygen atom (Hasnaoui *et al.*, 2005). Thus, in principle four products are possible, dependent on the nitrite and epoxide regioselectivity. In case of oxygen attack on the epoxide, a nitrite ester is produced that is unstable and decomposes to diol and nitrite by hydrolysis. In this way, the net reaction catalyzed by the halohydrin dehalogenase becomes epoxide hydrolysis. The nice enantioselectivity of halohydrin dehalogenase, usually with preferred conversion of the (*R*)-epoxide, makes it possible to perform kinetic resolutions of epoxides with these enzymes if nitrite is added.

D. Engineering of Halohydrin Dehalogenases

To exploit the attractive catalytic potential of halohydrin dehalogenases, attempts have been made to improve the enzyme for certain process conditions and applications. Tang *et al.* (2002) obtained mutants of halohydrin dehalogenase that are resistant to sulfhydryl oxidation. It appeared that especially Cys153 can undergo oxidation resulting in intermolecular disulfide bond formation and possibly other reactions. A Cys153Ser mutant showed improved stability and enhanced performance in the kinetic resolution of 2-*para*-nitrophenyl-2-bromoethanol. Other mutations that have been found to improve halohydrin dehalogenase catalytic properties are Trp249Phe and substitution of residues that form the halide-binding site. Mutation W249F improved the enantioselectivity and/or the catalytic rate, dependent on the substrate. The enantioselectivity was remarkably increased for the ring closure of 2-*para*-nitrophenyl-2-bromoethanol as well as for epoxide ring-opening reactions with nitrite (Tang *et al.*, 2005).

Directed evolution of halohydrin dehalogenase has been carried out by Davis *et al.* (2005), aiming to improve the HheC against product inhibition and harsh reaction conditions. Multiple rounds of random mutagenesis and gene shuffling were applied. This yielded an enzyme variant with improved productivity in the conversion of ethyl (*S*)-4-chloro-3-hydroxybutyrate (CHB) to ethyl (*R*) 4-cyano-3-hydroxybutyrate (see below). The mutations include the Cys153Ser substitution that was identified to be of importance on the basis of biochemical experiments (Tang *et al.*, 2002) and a number of surface mutations that likely contributed to enhanced stability by reducing unfavorable interactions. Other mutations are closer to the active site and influence the activity. Details about their properties have not been published.

E. Halohydrin Dehalogenases in Tandem Reactions

An important challenge in biocatalysis is the development of tandem or cascade reactions, in which multiple steps take place in the same reactor without the need for product isolation after each step. The possibility to use halohydrin dehalogenase for such a sequence was already suggested by Geigert *et al.* (1983), who proposed that the enzyme could ring-close halohydrins that were formed by haloperoxidase reactions to epoxides. Another example is the ring closure of a halohydrin to an epoxide, which, as mentioned above, can be drawn to completion by adding epoxide hydrolase.

A further tandem reaction, using a single enzyme to obtain a dynamic kinetic resolution, was studied by Lutje Spelberg *et al.* (2004) (Fig. 4A). As mentioned above, HheC was used to racemize epibromohydrin and

enantioselectively convert the (*R*)-enantiomer to (*S*)-3-azido-1-bromo-2-propanol. The product was obtained at >99% ee and good yield. The high ee was partially due toa product-polishing effect that occurred because the nonwanted (*R*)-enantiomer of the product was rapidly converted by the same dehalogenase to the azidohydrin, whereas the favored (*S*)-3-azido-1-bromopropanol was not converted and increased in enantiopurity.

Another interesting tandem reaction relying on enantioselectivity of halohydrin dehalogenase was studied by Majerić Elenkov *et al.* (2006b) (Fig. 4B). Starting with racemic methyl 4-chloro-3-hydroxybutyrate, the HheC enzyme produced methyl (*S*)-4-cyano-3-hydroxybutyrate and the remaining methyl (*S*)-4-chloro-3-hydroxybutyrate in high ee and good yield. In the cyanation reaction, the epoxide is formed as an intermediate, but it hardly accumulates due to rapid enzyme-catalyzed reaction with cyanide. The conversion was especially effective when the Trp249Phe mutant with enhanced enantioselectivity was used.

F. Statin Side-Chain Synthesis

Cholesterol-lowering agents of the statin family represent the largest family of drugs in terms of market volume (US13×10^9 per year), and the development of new synthetic routes is a highly competitive field. The possibility to use halohydrin dehalogenase for producing statin side chain has been studied by Codexis (Davis *et al.*, 2004). The process starts with the enantioselective ketoreductase-mediated reduction of 4-chloro-3-oxobutyrate ethylester, yielding ethyl (*S*)-4-chloro-3-hydroxybutyrate. This compound is converted by halohydrin dehalogenase to ethyl (*R*)-4-cyano-3-hydroxybutyrate, an important building block for the statin side chain (Fig. 5C). This conversion implies that the mutated dehalogenase is used to convert the (*S*)-enantiomer of the starting halohydrin via the (*S*)-epoxide, both of which are poorly converted by the wild-type enzyme because of its high enantioselectivity toward (*R*)-chlorohydrins and epoxides. The enantiopurity of the final product is obtained by the stereoselectivity of the ketoreductase, thus preventing the need for a kinetic resolution with its limitation of 50% maximum yield. Cofactor regeneration is done with glucose dehydrogenase or formate dehydrogenase. The enzymatic conversion of the halohydrin to the nitrile by the dehalogenase is more attractive than chemical conversion because it gives fewer side products. The tandem dehydrogenase–dehalogenase process can be carried out in a singe reactor vessel, with substrate concentrations of 100 g liter^{-1} or higher. An engineered halohydrin dehalogenase variant derived from HheC-with improved catalytic properties, as described above, is used for these

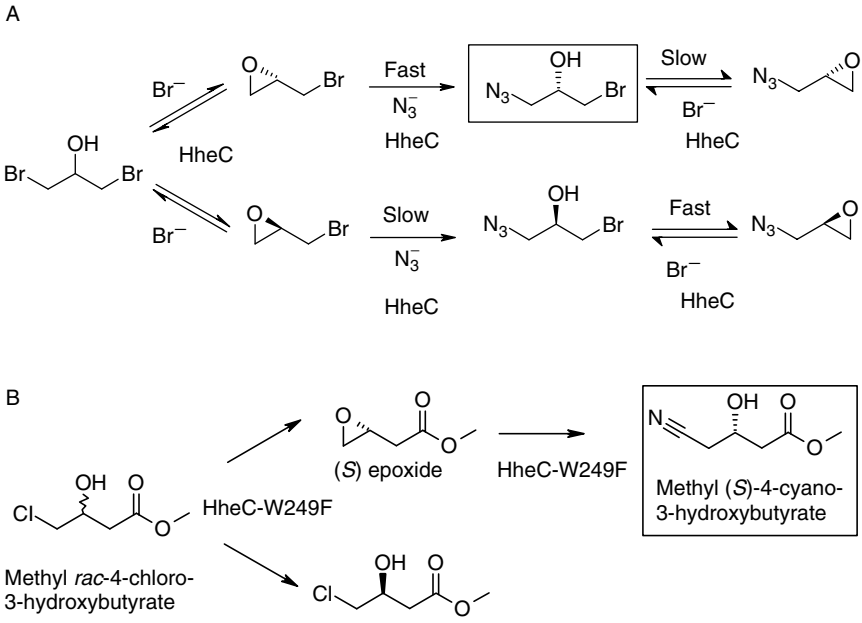

FIG. 4. Single-enzyme tandem reactions catalyzed by halohydrin dehalogenase. (A) Dynamic kinetic resolution of epibromohydrin to provide enantiopure (*S*)-1-azido-3-bromo-2-propanol. (B) Sequential kinetic resolution for producing a cyanohydrin from a chlorohydrin, in this case leading to methyl (*S*)-4-cyano-3-hydroxybutyrate.

conversions (Davis *et al.*, 2005). The process has been performed at Lonza at ton scale, and it is an interesting addition to a variety of chemoenzymatic routes that have been developed for obtaining statin side chains (Müller, 2005; Thayer, 2006).

V. Conclusions

Dehalogenases comprise a diverse group of enzymes belonging to different phylogenetic and mechanistic classes. They have biotechnological potential as a result of their high-catalytic power in the cleavage of carbon–halogen bonds of toxic environmental pollutants. Due to their enantioselectivity, dehalogenases can also be applied in biocatalysis for the kinetic resolution of chiral substrates. Furthermore, halohydrin dehalogenase can be used for epoxide ring opening and for replacement of halogen groups in *vicinal* haloalcohols by substituents such as azide, cyanide, and nitrite. Full-scale applications of dehalogenases have been

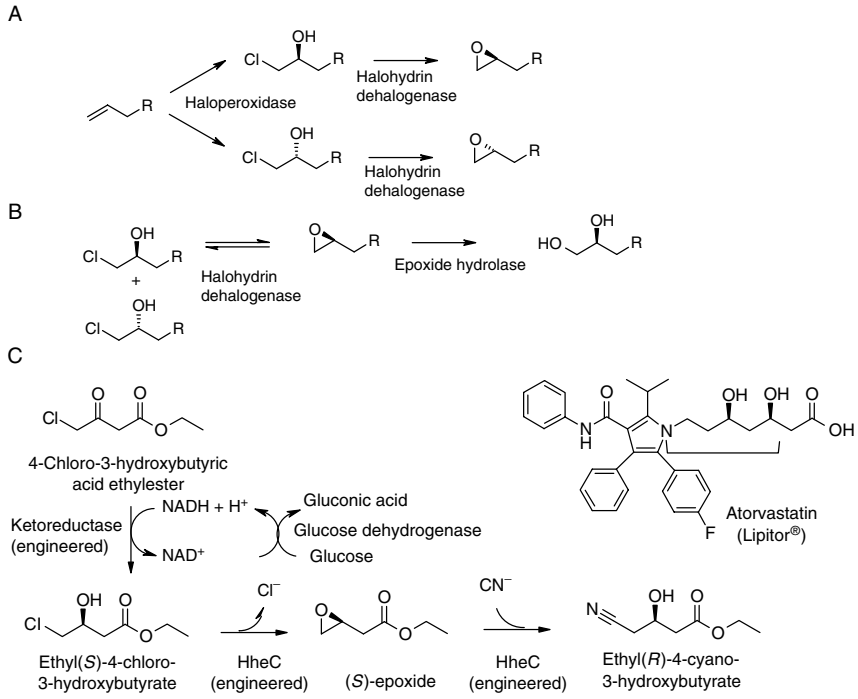

FIG. 5. Two- and three-enzyme tandem reactions involving halohydrin dehalogenase. (A) Haloperoxidase–halohydrin dehalogenase reaction proposed by Geigert *et al.* (1983). (B) Tandem reaction with epoxide hydrolase to draw the kinetic resolution of a halohydrin to completion (Lutje Spelberg *et al.*, 1999). (C) Process for the manufacture of a statin side-chain building block, as developed by Davis *et al.* (2004, 2005), using an enantioselective ketoreductase in combination with glucose dehydrogenase for cofactor recycling to produce ethyl (*S*)-4-chloro-3-hydroxybutyrate, and a halohydrin dehalogenase engineered by directed evolution to convert the product further to ethyl (*R*)-4-cyano-3-hydroxybutyrate. The structure of the high-volume drug atorvastatin is shown with the part of the side chain that can be produced via the halohydrin dehalogenase process indicated.

realized for kinetic resolution of 2-chloropropionic acid with D-haloacid dehalogenase producing cells, and of 2,3-dichloro-1-propanol and 3-chloro-1,2-propanediol with halohydrin dehalogenase producing cells. Furthermore, halohydrin dehalogenase can be used for preparation of the important statin intermediate ethyl (*R*)-4-cyano-3-hydroxybutyrate. New conversions have recently been found by replacing the nucleophile in halohydrin dehalogenase-catalyzed epoxide ring opening. It is likely that the diversity of dehalogenases is much broader than recognized to date. For biocatalysis, halohydrin dehalogenases of the SDR-family of enzymes seem to be most attractive, especially because of their

enantioselectivity, applicability in tandem reactions, and capacity to form C–C bonds when cyanide is used as the nucleophile.

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Lipases from Extremophiles and Potential for Industrial Applications

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I. Introduction

Lipases (E.C. 3.1.1.3), produced by many different microorganisms as well as by various eukaryotes, are carboxyl ester hydrolases of the α/β hydrolase superfamily (http://www.bmm.icnet.uk/supersites/foldlist.html; Cousin *et al.*, 1997), which catalyze the hydrolysis and synthesis of long-chain acylglycerols (Fig. 1) (Ferrato *et al.*, 1997; Sarda and Desnuelle, 1958). The size of lipases varies with the type of lipase and includes enzymes as small as 19.4 kDa (Kawasaki *et al.*, 2002) and oligomeric forms of above 300 kDa with subunits around 50 kDa (Salameh, 2006). Presently, eight classes of lipases are recognized

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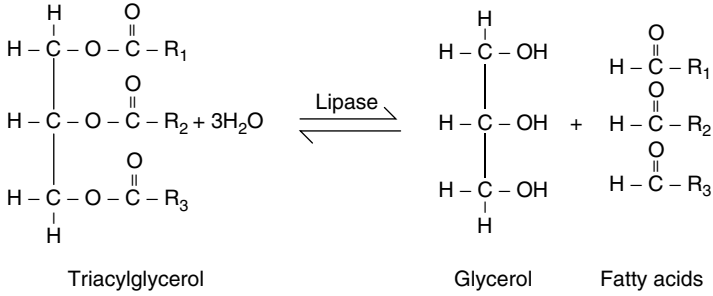

FIG. 1. Reaction catalyzing hydrolysis of lipids into glycerol and fatty acids in aqueous solution and reverse synthesis reaction occurring in the water-restricted organic solvent.

(Arpigny and Jaeger, 1999). Genes from over 60 bacterial lipases have been cloned and sequenced (Jaeger *et al.*, 1999).

There are interesting features that distinguish lipases from other hydrolases and especially esterases. Generally, esterases exhibit Michaelis–Menten kinetics and react with soluble substrates (triacylglycerols with short-chain fatty acids; e.g., triacetin). They have low activities toward insoluble glycerides (triacylglycerols with long-chain fatty acids; e.g., trilaurin). The maximum reaction rate is reached well before the saturation point (the maximum concentration of dissolved monomer) (Fig. 2). In contrast, true lipases are activated at the water–lipid interface, they show little activity when the substrate is in the monomeric form and their activity increases dramatically above the solubility limit where lipids start to form emulsions. This fact has led to the emergence of a phenomenon known as interfacial activation, which describes emulsions as a necessity for the lipolytic reaction to occur (Ferrato *et al.*, 1997; Sarda and Desnuelle, 1958). As a result of this, lipases exhibit with the long-chain acylglycerols substrates nonlinear enzyme kinetics (non-Michaelis–Menten kinetics).

Not all lipases show interfacial activation. Some of them, such as the lipases from the neutrophilic mesophilic *Pseudomonas aeruginosa* and *Burkholderia glumae* (bas. *Pseudomonas glumae*), have an amphiphilic lid covering their active sites (Verger, 1997), while others such as the lipases from *Bacillus subtilis* 168 (Lesuisse *et al.*, 1993), *Fusarium solani* (cutinase with lipolytic activity) (Hjorth *et al.*, 1993), and guinea pig pancreas (Martinez *et al.*, 1992) have no lid.

The discoveries of these exceptional lipases led to the conclusion that the presence of a lid domain and interfacial activation are unsuitable criteria to classify an enzyme as a lipase. Therefore, the working definition is true lipases are carboxylesterases that catalyse the hydrolysis of long-chain acylglycerols (Verger, 1997).

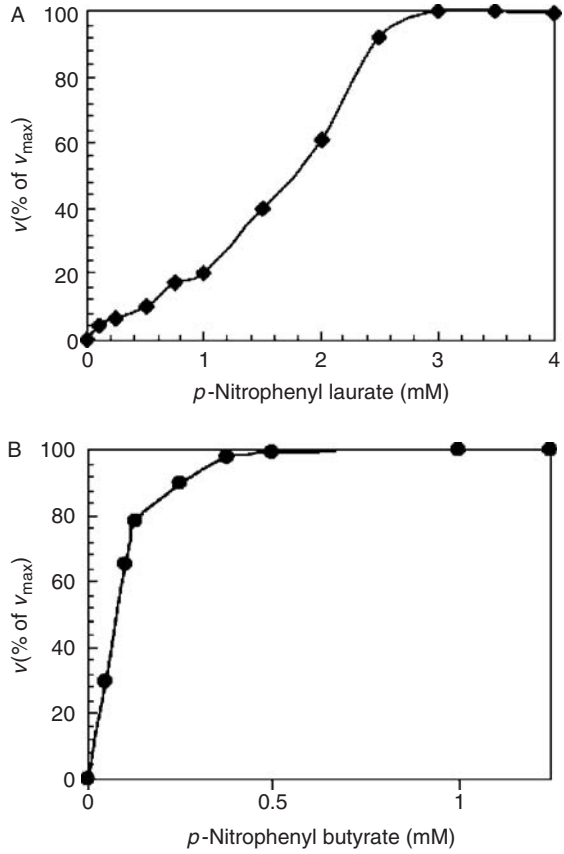

FIG. 2. The effect of substrate concentration on hydrolysis rate. *Thermosyntropha lipolytica* lipase, LipA (A) and *Alicyclobacillus acidocaldarius* esterase, EST2 (B) (adapted from Chahinian *et al.*, 2005). The activity is expressed as percentage of maximal observed activity (v_{max}).

II. Lipases from Extreme Microorganisms

Extremophiles includes organisms able to thrive at extremes of temperature, pressure, low water activity, salinity, acidity, or alkalinity or are resistant to exposure of high radiation, pressure, metal concentrations, or organic solvents and combinations thereof. Extremophiles can be not only found in niches exhibiting these conditions but several have been isolated from nonextremophilic environments (Engle *et al.*, 1996; Kevbrin *et al.*, 2004; Wiegel, 1998, 2002).

Extremophiles have the potential to produce uniquely valuable biocatalysts that function under conditions in which usually the enzymes of their nonextremophilic counterparts could not. However, also a limited number of lipases that function at extreme conditions were isolated from mesophilic origin, for example, thermophilic lipase from *Burkholderia cepacia* (Rathi *et al.*, 2000), acidophilic and thermophilic lipase from *Kurtzmanomyces* sp. I-11 (Kakugawa *et al.*, 2002), and alkaliphilic lipase from *Bacillus subtilis* 168 (Lesuisse *et al.*, 1993). Another interesting example is the lipolytic *Pseudomonas aeruginosa*, a proteobacterium usually not regarded as an extremophile, but which was isolated growing in 99% of a triglyceride and 1% water phase at 42°C. Growth ceased when the water was used up but continued again when additional 1% water aliquots were added thus can be regarded as extremophile (Shabtai, 1991). The 40-kDa purified lipase had about a tenfold higher activity in organic solvents than in watery solution, but otherwise had a typical pH = 7.0 range but a markedly high thermostability in organic solvents beyond 30 days at up to 55°C (Shabtai and Baya-Mishne, 1992).

On the basis of comparison of their amino acid sequences and some fundamental biological properties, bacterial lipases were classified into eight families (Arpigny and Jaeger, 1999). Most psychrophilic lipases and esterases, such as the ones from *Moraxella* sp., *Psychrobacter immobilis*, and *Pseudomonas* sp. B11–1 belong to family IV [sequence similarity to mammalian hormone-sensitive lipase (HSL)] and to family V. Whereas most thermophilic lipases with similar properties from *Geobacillus* (bas *Bacillus*) *thermocatenulatus*, *G. stearothermophilus*, and *G. thermoleovorans* (Cho *et al.*, 2000; Kim *et al.*, 1998; Rua *et al.*, 1997; Schmidt-Dannert *et al.*, 1994; Sinchaikul *et al.*, 2001) belong to subfamily 5 of family I ''the true lipases.'' The *Sulfolobus shibatae* thermostable carboxyesterase hydrolyzing only C_2–C_{16} esters, however, belongs to the HSL family (Ejima *et al.*, 2004).

Here, we summarize the properties of lipases from mainly two types of extremophiles, that is, psychrophiles (i.e., growing optimally at or below 15°C) and thermophiles growing optimally at elevated temperatures of above 50°C, as well as summarize the distribution of lipase activity in halophiles. Psychrophilic lipases or lipase activity at temperatures below 18°C were frequently detected in marine organisms from (ant)arctic waters; the organisms are also slightly salt tolerant growing up to 6% (w/v) NaCl and with growth optima around 2–3% (w/v). Many members of extremophilic Bacteria and Archaea produce carboxylesterases (so-called C_4 esterases and C_8 ester lipases compared to true lipase activity hydrolyzing fastest triglycerides containing fatty

acids of 12–18 (20) carbons = called also C_{14}–C_{20} lipases). However, a few enzymes have a wide substrate spectrum such as the enzyme from *Geobacillus thermocatenulatus* hydrolyzing *p*-nitrophenyl ester with C_4–C_{18} but with highest activity toward *p*-nitrophenyl caproate (C_{10}) and toward tributyrin (C_4 acyl group) but also being able to hydrolyze triolein (C_{18} acyl groups) (Rua *et al.*, 1997; Schmidt-Dannert *et al.*, 1996). The authors regard such enzymes more as esterase-lipases (also called C_8 activity). This chapter deals with those carboxylesterases hydrolyzing lipids containing long-chain fatty acids (=true lipases). They are less common than esterases and esterase-lipases. For instance, Cornec *et al.* (1998) tested 160 thermophilic and hyperthermophilic isolates from deep-sea hydrothermal vents of which 47 were esterase positive (i.e., hydrolyzing C_8-esters) but none was able to hydrolyze $C_{16/18}$ carbon fatty acid containing glycerides. So far, no true lipases have been purified and characterized from a thermophilic *Archaeum*, although the presence of archaeal lipases has been described in several publications (Bhatnagar *et al.*, 2005 and literature cited there in). The closest to an archaeal lipase is the characterization of the 33-kDa *Sulfolobus shibatae* and the 35.5-kDa monomeric *Archaeoglobus fulgidus* thermostable carboxyesterase hydrolyzing C_2–C_{16} esters and showing similarities to the HSL family with a nonlinear kinetic behavior (Ejima *et al.*, 2004; Manco *et al.*, 1999). Also, to the knowledge of the authors, no true (extreme) halophilic lipase has been purified despite that lipase activity has been demonstrated in many true (extreme) halophiles. Lipases from halophiles including from the novel group of haloalkalithermophiles could be of great interest. The alkaliphilic lipases have been reviewed in the past (Horikoshi, 1999; Ito *et al.*, 1998; Jaeger and Eggert, 2002; Zheng, 2001).

To present some of the general properties of lipases and industrial applications, examples of lipases from mesophiles had to be included. The present status can be summarized by that in several instances lipases from extremophiles would have an advantage; however, either the lipases from extremophiles for the specific application-oriented specification have not been purified and characterized or not been optimized for the application, their genes not cloned nor their economical production undertaken.

A. Lipases from Psychrophiles (Cold Active Enzymes)

On the basis of market analyses, the detergent industry has made a shift to seek lipases from psychrophilic organisms, since washing at low temperature will save energy and lower the cost, and make it affordable

to less industrialized countries, including rural India and China. One of the first lipase producing psychrophilic bacteria was a lipolytic *Acinetobacter* sp. (Breuil and Kushner, 1975). Several psychrotolerant lipolytic *Moraxella* species were subsequently isolated from the Antarctic seawater, they all produce lipase that have high activity, but not an optimum in the temperature range of 10–20°C (Feller *et al.*, 1990a). Consequently, three lipases genes from *Moraxella* TA144 were sequenced and cloned in *Escherichia coli* (Feller *et al.*, 1990b, 1991a,b). This was followed by isolation of many other psychrophilic lipolytic bacteria including *Psychrobacter immobilis* B10 (Arpigny *et al.*, 1993), *Pseudomonas* sp. B11–1 (Choo *et al.*, 1998), *Acinetobacter calcoaceticus* LP009 (Pratuangdejkul and Dharmsthiti, 2000), *Arthrobacter flavus* CMS19YT (Reddy *et al.*, 2000), *Pseudomonas fragi* (Alquati *et al.*, 2002), *Psychrobacter okhotskensis* (Yumoto *et al.*, 2003), and the psychrotolerant bacterium *Corynebacterium paurometabolum* MTCC 6841 (Joshi *et al.*, 2006) (Table I). However, most of these lipases were not characterized in detail or resembled more enzymes from mesophilic microorganism and were lacking maximal activities below 20°C.

One factor that contributes to the efficient catalytic activity of psychrophilic enzymes at lower temperature is the increase in the enzyme's flexibility. Arpigny *et al.* (1993) first reported on a sequence

TABLE I

LIPASES ACTIVE AT LOW TEMPERATURES[a]

Source	T (°C)[b]	pH_{optima}	References
Pseudomonas sp. B11–1[c]	5–35[d]	8.0	Choo *et al.*, 1998
Moraxella TA144	10–20[d]		Feller *et al.*, 1990a
Pseudomonas sp. KB700A[e]	10–30 (30-min assay)	8.0	Rashid *et al.*, 2001
Pseudomonas fluorescens B68	5–30[f] (10-min assay)	8.0	Luo *et al.*, 2006
Corynebacterium paurometabolum MTCC 6841	25 (10-min assay)	8.5	Joshi *et al.*, 2006

[a]To the authors best knowledge, there is no true psychrophilic lipase with optimum activity ≤10°C.
[b]Low temperature range with decent catalytic activity but not necessarily maximum.
[c]Maximum activity observed was at 45°C.
[d]Assay time was not mentioned.
[e]Maximum activity observed was at 35°C.
[f]Maximum activity observed was at 20°C with 55% relative activity at 5°C.

modifications which were later also seen in other enzymes and are characteristic for psychrophilic lipases (Choo *et al.*, 1998). In comparison to thermophilic and mesophilic proteins, the number of salt bridges, ionic interactions, hydrogen bonds, hydrophobic, and/or intersubunits interactions are usually lower in psychrophilic proteins (Bentahir *et al.*, 2000; Fields, 2001; Gerday *et al.*, 1997; Kim *et al.*, 1999). On the basis of the interest of the laundry detergent industry, it is expected that in the near future more and true cold active lipases will be discovered and described.

B. Lipases from Thermophiles (Thermoactive Lipases)

The most investigated enzymes from extremophiles are those from thermophiles. These enzymes are generally stable at high temperatures and in organic solvents (Ejima *et al.*, 2004; Fucinos *et al.*, 2005; Li and Zhang, 2005; Pantazaki *et al.*, 2002). Several of the lipases have been heterologously expressed. An interesting system for expressing and enhanced secretion into the media for the *Geobacillus stearothermophilus* L1 lipase was published by Ahn *et al.* (2004) using *Saccharomyces cerevisiae* and a translational fusion to cellulose-binding domain. The fusion increased the excretion of the thermophilic lipase sevenfold. Furthermore, the cellulose-binding domain can also then be used as affinity tag for purification. Interestingly, compared to other enzymes from mesophiles, many of the lipases from mesophiles exhibit a relative high-temperature optimum and stability in organic solvents. However, only few lipases from mesophiles withstand temperatures above 75°C for an extended time. One of those is the unusual lipase from *Burkholderia cepacia* which has maximum activity at 90°C (Rathi *et al.*, 2000), another one is the lipase from the yeast *Kurtzmanomyces* sp. I-11 with optimum temperature for activity at 75°C using a 15-min assay, and with a pH activity range from 1.9 to 7.2, and an optimum at pH 2–4, it is also one of the most acidophilic lipases (Kakugawa *et al.*, 2002). In its pH characteristics, it is very similar to the interesting acidothermotolerant enzymes from *Geobacillus stearothermophilus* SB-1 and *Bacillus licheniformis* SB-2. The enzymes retain 70% and 50% of olive oil hydrolyzing activity, respectively, when incubated at 50°C and acidic pH 3.0. The SB-1 lipase exhibit a half-life of 25 min at 100°C and pH 3.0, but only of 15 min at pH 6.0 (Bradoo *et al.*, 2004). Another highly thermostable lipase was reported by Hashwa and Al-Khudary (2002) for the 80-kDa lipase from a thermophilic *Thermoactinomyces* sp., withstanding at neutral pH boiling without loosing activity above 2 hours.

Thermophilic lipases (Table II) serve as excellent models for understanding protein stability as well as they have a significant potential for biotechnology. Factors that can contribute to the high thermostability of a given enzyme include changes in amino acid residues, increased salt-bridge content, reductions in cavity size, increased hydrophobic interactions, and changes in solvent-exposed surface areas (Adams and Kelly, 1998; Demirjian *et al.*, 2001; Eichler, 2001). Although several lipases from mesophilic microorganisms are to some extent thermostable and alkali stable, enzymes produced by thermophilic, alkali tolerant bacteria are generally more thermostable at alkaline pH while exhibiting high specific activities at elevated temperature (>70°C) and alkaline pH (>9.0) (Table II). While true lipases have been reported from aerobic thermoalkaliphilic bacteria (Bell *et al.*, 1999; Kim *et al.*, 1998, 2000; Lee *et al.*, 1999; Rua *et al.*, 1997), the characterization of a lipase from an anaerobic thermophilic bacterium (*Thermosyntropha lipolytica*) occurred only recently (Salameh, 2006). In addition, Antranikian reported at the Thermophile 2005 Meeting, the isolation of thermophilic lipases from *Thermoanaerobranka gottschalkii* (Prowe and Antranikian, 2001).

Thermosyntropha lipolytica was isolated with a specific intent of finding bacterial lipases being active at elevated temperature under commercial washing conditions, that is, in the presence of washing detergent having alkaline pH (Svetlitshnyi *et al.*, 1996). Many of the thermostable lipases can tolerate detergents or are only slightly inhibited pending on the different conditions employed. In contrast, the commercial Lipolase® activity is strongly reduced in the presence of detergent, although used in commercial laundry detergents. The addition of 0.1% Triton X-100 plus linear alkyl benzene sulfonate or the laundry detergent TIDE increased the specific activity more than threefold (Svetlitsnyi, unpublished results). In contrast to most lipolytic microorganisms, *Thermosyntropha lipolytica* does not utilize the liberated glycerol from the lipids, but, in syntrophic coculture with *Methanobacterium* sp. or thermophilic sulfate reducers, uses the liberated long-chain fatty acids as carbon and energy source (Svetlitshnyi *et al.*, 1996).

Several anaerobic bacteria which are able to utilize fatty acids in syntrophic relationship have been isolated and characterized (Lorowitz *et al.*, 1989; McInerney *et al.*, 1979, 1981; Roy *et al.*, 1986; Stieb and Schink, 1985; Zhang *et al.*, 2004). *Syntrophothermus lipocalidus* DSM 12680^T is the only other syntrophic thermophilic bacterium recently isolated; however, it is not described as a lipase producer, since the strain did not utilize any triglycerides (Sekiguchi *et al.*, 2000). To the

TABLE II

THE MOST THERMOPHILIC LIPASES

Source	$T_{max\ act.}$ (°C)	pH_{optima}	Stability	References
Thermosyntropha lipolytica	96 (20-min assay)	9.0–9.6	LipA: $t_{1/2}$ 6 hours at 100°C, 24 hours at 75°C. LipB: $t_{1/2}$ 2 hours at 100°C, 24 hours at 75°C at pH 8	Salameh, 2006
Burkholderia cepacia	90[a]	11	$t_{1/2}$ 13 hours at 90°C	Rathi *et al.*, 2000
Geobacillus thermoleovorans	75 (5-min assay)	8.0	$t_{1/2}$ = 1 hour at 60°C at pH 7.5	Cho *et al.*, 2000
Bacillus sp. THLO27	68–70 (30-min assay)	7.0	$t_{1/2}$ = 2 hours at 60°C and 75°C at pH 8.0	Dharmsthiti and Luchai, 1999
Geobacillus thermocatenulatus	60–70[a]	BTL2 8–9	$t_{1/2}$ = 30 min at 60°C at pH 9.0	Rua *et al.*, 1997
Geobacillus stearothermophilus L1	60–65 (5-min assay)	9–10	Retains 30% activity after 30 min at 65°C at pH 8.0	Kim *et al.*, 1998
Myroides odoratimimus (*bas Flavobacterium odoratum*)	60	10.0	$t_{1/2}$ = 10.2 min at 70°C at pH 8.5	Labuschagne *et al.*, 1997
Bacillus sp. J33	60 (30-min assay)	8.0	$t_{1/2}$ = 12 hours at 60°C	Nawani and Kaur, 1999
Bacillus sp. TG4	60 (1-min assay)	9.0	Retains 80% activity after 30 min at 60°C	Bell *et al.*, 1999
Geobacillus stearothermophilus P1	55 (5-min assay)	8.5	$t_{1/2}$ = 2 hours at 65°C at pH 8.5	Sinchaikul, 2001

[a]Assay time was not mentioned.

knowledge of the authors, besides the above mentioned two bacteria, no other thermophilic anaerobe able to utilize long-chain fatty acid triglycerides has been published or characterized.

Thermosyntropha lipolytica constitutively produces two lipases, LipA and LipB, that show maximal activity at 96°C using a 20-min assay (Fig. 3) and retain 50% activity after 20-hour incubation at 75°C (Salameh, 2006). Beside the above mentioned enzyme from *Thermoactinomyces* sp., the only other lipase with such high thermal activity and stability was surprisingly found in the mesophilic *Burkholderia cepacia* (Rathi *et al.*, 2000). However, comparing the physical properties of these lipases is hampered by the different assay substrates and procedure used in the two laboratories. As a comparison, the most stable esterase is the one from *Pyrococcus furiosus* with an optimal activity around 100°C and a half-life of 50 min at 126°C (Ikeda and Clark, 1998). Both above lipases prefer glycerides with long-chain fatty acids, with maximum activity exhibited toward trioleate ($C_{18:1}$), and prefer to hydrolyze ester bonds at position 1 and 3 of triglycerides (Salameh, 2006). This specificity is similar to that exhibited by other lipases such as those from the thermophilic *Bacillus* sp. THL027 (Dharmsthiti and Luchai, 1999), *G. thermocatenulatus* (Schmidt-Dannert *et al.*, 1994), mesophilic *Bacillus subtilis* 168 (Lesuisse *et al.*, 1993), and from some mesophilic Gram type negative bacteria (Lee *et al.*, 1993; Matsumae and Shibatani, 1994).

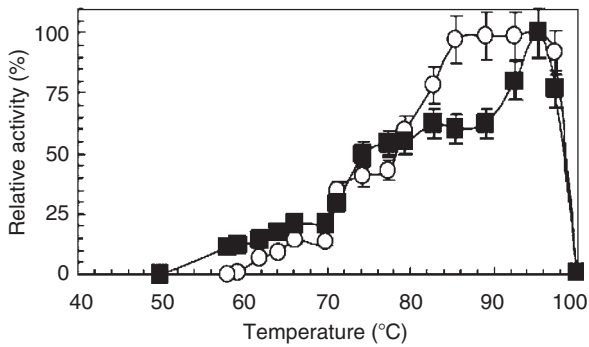

FIG. 3. Effect of temperature on *Thermosyntropha lipolytica* LipA (○) and LipB (■) activity. Lipase was assayed spectrophotometrically using *p*-nitrophenyl laurate (*p*NPL). The assay was typically carried out for 20 min at 96°C, and the A_{405} of the liberated *p*-nitrophenol was determined. A 100% relative activity was 11.8 ± 0.5 U·mg^{-1} and 13.0 ± 0.6 U·mg^{-1} for LipA and LipB, respectively. Activity values were corrected for controls without enzyme.

Whereas the lipase from *Thermosyntropha lipolytica* was expressed constitutively (expressed in the absence and presence of triglycerides) with little variation in activity per cell density, other lipases expression are more regulated. Becker *et al.* (1997) described in detail the influence of dilution rate in a continuous culture on the lipase production by the thermophilic *Bacillus* sp. IHI-91; the higher the dilution rate, the lower the lipase activity. Olive oil was the best inducers compared to other oils such as fish sunflower and soybean oils, about 70% of the activity was associated to the cells and residual substrate.

One of the most important industrial lipase applications is the stereo- and enantiomeric-specific organic synthesis. LipA and LipB were successfully used to catalyze a variety of etherification reactions in isooctane at 85°C (Salameh, 2006), including fatty acids alcohol and diacylglycerols (Fig. 4). As a result of their high-thermal activity and stability and based on some assays, these thermophilic enzymes could be used for the following applications: (1) the production of fatty acids at elevated temperatures from beef tallow; (2) the production of laundry detergent additives for high temperature washing based on their high thermostability,

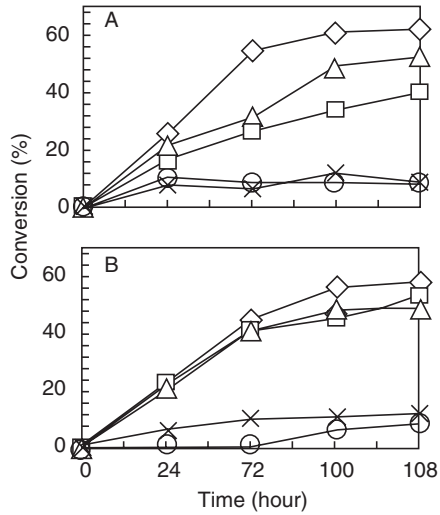

FIG. 4. Time course of diacylglycerol (DAG) synthesis catalyzed by LipA (A) and LipB (B). 1,3-Dioleoyl glycerol (◇), 1-oleoyl-3-lauroyl glycerol (□), 1-oleoyl-3-octoyl glycerol (△), 1,3-dilauroyl glycerol (×), and 1-lauroyl-3-oleoyl glycerol (○). Synthesis of DAG were conducted in 10-ml serum bottles that contain 100 μg of lyophilized LipA and LipB, 100-mM monoacylglycerols, and 100 mM of fatty acids at 85°C for 100 hours. The unesterified free fatty acids were measured using the NEFA C kit (Waco, United States).

thermoactivity, and tolerance against commercial detergent components; (3) the production of diacylglycerols; and (4) the production of various fatty acid esters at elevated temperature which might provide a solution for the low substrate solubility in organic solvents.

Most of the lipases that are commercially adopted by many companies are from *Pseudomonas* sp., and this is due to the fact that these lipases are the first to be isolated, cloned, and characterized, their three-dimensional (3D) structures among the earliest to be revealed along with well-established biochemical and genetic data. Moreover, most of these lipases were gone through a variety of molecular modifications to produce effective enzymes with more desirable characteristics (Gupta *et al.*, 2004; Jaeger and Reetz, 1998).

C. Lipases from Halotolerant/Halophilic Microorganisms (Salt-Tolerant Enzymes)

For many halotolerant, moderate, and extreme halophilic members of the Bacteria and Archaea domains, ''lipolytic activity'' has been included in the list of the published properties. However, in several instances this means the bacteria were able to hydrolyze Tween 40, 60, or 80 (also called polysorbate). Tween 40, 60, and 80 are polyoxyethylensorbitan ester of palmitate, stearate, and monooleate, respectively. Tween 85 (the polysorbate trioleate ester) is rarely tested. In addition, the API ZYM test kit is used, which differentiates between hydrolysis of C_4-esters (esterase activity) C_8-ester (ester-lipase activity) and C_{14}-ester (lipase activity). Although in some cases they only hydrolyze the C_8-ester, they are still called lipolytic. Usually hydrolysis of Tween 80 and 85 correlates with the presence of lipases able to hydrolyze glycerides with C_{18} fatty acids; however, exceptions exist. Lipolytic halophiles are found among different phyla of anaerobic and (facultative) aerobic Bacteria and Archaea. Many belong to the phyla *Proteobacteria*, especially (facultative) aerobic alpha and gamma *Proteobacteria*, but includes also bacteria from the delta branch such as strain from the *Myxobacteria–Bdellovibrio* branch (Baer *et al.*, 2004). Most of the bacteria were isolated from marine environments and are facultative or moderate halophiles which are frequently also adapted to cold temperatures, thus being double extremophiles (e.g., *Psychrobacter okhotskensis* from the Okhotsk Sea, having C_4, C_8, and C_{14} esterase/lipase activity and Tween 20–80 activity in 0–10% NaCl containing media and growing between 0 and 36°C; Yumoto *et al.*, 2003). Many of the other isolates are only tolerating slightly above seawater level salt concentrations, thus those lipases are probably not very salt tolerant (e.g., *Marinobacter*

flavimaris growing between 2–6% (w/v) NaCl C_{14} lipase activity; Yoon *et al.*, 2004). From saline lakes, moderate or true halophiles such as the thermotolerant *Psychroflexus tropicus*, belonging to the Cytophaga-Flavobacterium-Bacteroides group, have been isolated showing beyond the C_4/C_8 esterase also C_{14} lipase activity (Donachie *et al.*, 2004) or *Marinobacter lipolyticus* (Martin *et al.*, 2003) growing optimally at 7.5% NaCl. C_{14} lipolytic halotolerant/true halophilic *Firmicutes* have been isolated from salt lakes and marine saline ponds, for example, the actinobacteria *Yania* (Li *et al.*, 2005) and from salty soils, for example, *Isoptericola* growing optimally at 10% (w/v) NaCl and pH 8–9 (Zhang *et al.*, 2005) or *Sinococcus qinghaiensis* belonging to the *Bacillales*; growing between 1% and 25% (w/v) NaCl (Li *et al.*, 2006).

Archaea (true halophiles and extreme halophiles) have been described from various sources including from ancient, that is, >200-million-year-old rock salt such as *Halococcus dombrowski*, with C_8 ester-lipase activity (Stan-Lotter *et al.*, 2002). Bhatnagar *et al.* (2005) described lipolytic activity in Archaeal *Halomebacteria* (*Halobacteria*) growing at 2- to 4-M salt and exhibiting maximal lipase production at 4-M NaCl and pH 8.0 at 45°C. Unfortunately, none of the lipolytic activities have been further biochemically characterized in respect to salt requirement and resistance and influence of the salt on the substrate range and specificity. Already in 1970, Gonzalez and Gutierrez (1970) published a survey of lipolytic activity in halophilic microorganism, and pointed out the importance of halophilic lipases for biotechnology, a comment also mentioned in nearly all reviews on industrial halophilic enzymes and microorganisms (Margesin and Schinner, 2001); however, as far as the authors are aware of, the lipases of (extreme) halophiles either from Bacteria or Archaea have neither been purified, well characterized, nor their encoding genes sequenced. Due to the diversity of the gene sequences, it is more difficult to locate lipases in the existing genomes, although possible, for example Klenk *et al.* (1997), a lipase encoding gene from *Archaeoglobus*; Bell *et al.* (2002, 1999), environmental lipase sequences, leading to a lipase with less than 20% similarity to the amino acid sequence of other lipases; Rees *et al.* (2004), finding 1 lipase positive clones in 100,000 from an environmental library from a Kenyan soda lake and 1 positive in 30,000 clones from an olive oil enrichment. There is no question, lipases from archaeal extreme halophiles should be of great interest for nonaqueous synthesis, since these archaea uniquely accumulate intracellular high $MgCl_2$ concentrations in addition to KCl and NaCl (>3 M) to stabilize their enzyme. Since salt reduces the water activity for enzymes in extreme halophilic archaea, the lipases should be stable in mixtures of

water and organic solvent and in nonaqueous organic solvents. Furthermore the $MgCl_2$ enhances in cultures the lipolytic activity by removing the liberated fatty acids and thus preventing inhibition. This effect has been also shown for marine lipolytic bacteria (Bruni *et al.*, 1982). Troller and Stinson (1978) reported on the inhibitory effect of salt concentrations between 0.1 and 1.2 M corresponding to water activities between $\alpha_w = 0.995$ and 0.96 yielding 8–86% inhibition of the nonhalophilic lipase from *Staphylococcus aureus*. Furthermore, compatible solutes such as found in halophiles as well as synthetic ones have been shown to increase the lipase half-life when incubated at various temperatures for 10 min by 11–12°C (Vasurdevamurthy *et al.*, 2004). The synthetic homodeanol betaine was found to be the best stabilizer for the lipases for freezing at −30°C or for freeze-drying. The authors are convinced it is now just a question of time that a lipase from a (extreme) halophile will be described in detail.

III. Improving Lipases for Efficient Applications

For biocatalysts in general and lipases specifically, turnover rates and stability under the application conditions are the major issues that need to be improved. Lipases with potential for present day applications in the detergent industry have to be thermostable, alkali stable, stable against proteolytic action, oxidative compounds, and detergent ingredients and as said before, have to have good activity below room temperature, combined with low substrate specificity. A desire for kinetic and turnover rate changes is more likely needed in the food, chemical, and pharmaceutical areas. Enzymes for the combined enzyme-chemical synthesis of stereospecific compounds need to be stable in organic solvent and exhibit high stereospecificity in the reverse, that is, synthesis direction. The performance of an enzyme that is active in one given reaction is not always sufficient for its application in another industrial process. Consequently, there are various chemical, physical, and genetic modification strategies proposed and applied to enhance the properties and discover the principal suitability of a given lipase (Bornscheuer, 2005; Bornscheuer *et al.*, 2002; Villeneuve *et al.*, 2000). These strategies are also applicable to other biocatalysts. Some of these strategies include immobilization of the lipases in a defined space, that is enclosed by material barrier for physical separation of the enzyme from the reaction medium, and at the same time permeable to reactants and products: for example, microencapsulation using nanofibrous membranes (Ye *et al.*, 2006), reverse micelles (Carvalho and Cabral, 2000), or it could be achieved by attachment to a carrier either covalently (Yemul and Imae, 2005), by hydrophobic

interactions (Dosanjh and Kaur, 2002), by ion exchange adsorption, or by cross-linking (Kartal and Kilinc, 2006; Kilinc *et al.*, 2006). Mineral support such as glass beads (Marlot *et al.*, 1985), silica (Ivanov and Schneider, 1997; Wisdom *et al.*, 1985), and alumina (Brady *et al.*, 1986) have also been used. However, the most recent used supports are ion exchange resins, biopolymers (Gitlesen *et al.*, 1997; Gray *et al.*, 1990), and celite (Ivanov and Schneider, 1997; Kang and Rhee, 1988; Kilara and Shahanil, 1977; Svensson *et al.*, 1990). The vast majority of immobilization experiments were conducted on mesophilic lipases, mainly to enhance their stability and turnover ratio. However, the thermostability of a thermophilic lipase from *Bacillus coagulans* BTS-3 was improved by immobilization on silica (Kumar *et al.*, 2006). In general, immobilization of enzymes stabilizes them and renders them to be reusable biocatalysts since it facilitates downstream processing through easy separation processes. This leads to a significant reduction in the operating costs (Balcão *et al.*, 1996; Kilara and Shahanil, 1977).

Protein engineering by rational protein design using recombinant DNA technologies allows the amino sequence of the lipase to be changed so that it acquires different properties (specificity, selectivity, and stability) that can better fit a particular application (Bornscheuer and Pohl, 2001; Dalby, 2003). Below are a few examples of engineered lipases mainly from mesophiles, since they were the first to be characterized and commercially utilized. However, by the beginning of this century, researcher started to apply protein engineering methods on thermophilic lipases and esterases, for example Kauffmann and Schmidt-Dannert (2001) carried out a single round of random mutagenesis to improve the phospholipase activity of the thermoalkaliphilic lipase from *G. thermocatenulatus* BTL2. Moreover, error-prone PCR was used on two highly thermophilic carboxylesterases from the archaea *Alicyclobacillus acidocaldarius* and *Archaeoglobus fulgidus*, to modify the enzymes enantioselectivity to produce (*S*)-2-chloropropionic acid (Manco *et al.*, 2002).

Liebeton *et al.* (2000) dramatically enhanced the enantioselectivity of the lipase from *Pseudomonas aeruginosa* by performing successive rounds of random mutagenesis using error-prone PCR. Using a combination of error-prone PCR with DNA shuffling produced a lipase variant of *Pseudomonas aeruginosa* with a complete inversion of the enantioselectivity (Zha *et al.*, 2001). A combination of error-prone PCR and cell surface display were successfully used to enhance the activity of *Rhizopus oryzae* lipase for both hydrolysis and esterification reactions (Shiraga *et al.*, 2005). Another study focused on exploring the effects of altered active site accessibility and protein backbone

flexibility on the catalytic performance of lipase B from the yeast *Candida antarctica*. Although it is a mesophile from Antarctica, the lipase is not a low-temperature enzyme and has even been used for synthesis of heptyl ferulate at 90°C (Yoshida *et al.*, 2006). Qian and Lutz (2005) employed successfully circular permutation (relocating of the protein's N- and C-termini) for modification. Kinetic analysis indicated that a majority of the obtained enzyme variants either retained or surpassed wild-type activity on a series of standard substrates.

Kato *et al.* (2005) established a strategy for exploring functional proteins associated with computational analysis by using fuzzy neural network (FNN). FNN, a type of artificial neural network, automatically constructs complex model structures by learning the hidden relationship between input and output data, and it functions as a predictor. By using this approach, they successfully reversed the enantioselectivity of the wild-type lipase of *Burkholderia cepacia* KWI-56 from the (*S*)-configuration to the (*R*) form for the substrate *p*-nitrophenyl 3-phenylbutyrate. So far, this approach has not been applied to any lipase from a psychrophile or thermophile.

IV. Regio- and Stereospecificity of Lipases

Lipases have been found to exhibit varying degrees of specificity for the positioning of the ester bond on the alcohol backbone, referred to as regioselectivity. Although the glycerol molecule has plane symmetry, the two primary alcohol groups are sterically distinct, and substitution of these hydroxyl groups with different acyl groups leads to optically active compounds. The enantiopreference of a lipase is determined by the differences in size and the hydrophilic versus hydrophobic environment of the pockets around the catalytic site. Generally four substrate-binding pockets have been identified for triglycerides: three for the fatty acids bound at position sn-1, sn-2, and sn-3 and one oxyanion hole (Jaeger *et al.*, 1999). The glycerol molecule is conventionally written in the Fisher projection with the secondary alcohol group to the left and the two primary alcohol groups to the right, with the backbone carbons numbered 1, 2, and 3 from top to bottom. Many microbial lipases preferentially cleave the ester bonds at the primary alcohol positions (sn-1 and sn-3, for stereospecifically numbered glycerol), such as the thermophilic lipases LipA and LipB of *Thermosyntropha lipolytica* (Salameh, 2006), although many bacterial lipases can hydrolyze the ester bond at all three positions. Furthermore, there is usually specificity for

the chain length of fatty acid and the degree of saturation, and thus the regioselectivity of the enzyme may be more or less pronounced depending on fatty acid chain length (Jaeger *et al.*, 1994; Kazlauskas, 1994). The regiospecific lipases offer the greatest potential for industrial application, such as production of structured lipids with unique functional properties (Sonnet and Gazzillo, 1991). The stereo- and enantioselectivity enables the enzyme to discriminate between the enantiomers of a racemic pair. This property is very important in the synthesis of fine chemicals, because of its ability to produce at least a highly enriched active enantiomer instead of a mixed racemic compound.

Most lipases can act on several different kinds of acyl-alcohols, which include specificity as well for the alcohol molecule [e.g., LipA and LipB of *Thermosyntropha lipolytica* efficiently catalyzed the synthesis of octyl and lauryl oleate but not hexyl or butyl oleate (Salameh, 2006)]. The reverse, that is, synthesis reaction is energetically favored in water-restricted environments with enzyme specificities and preferences largely retained. Thus, the synthesis of specific acyl-alcohol molecules can be envisioned by setting the correct conditions in the aqueous solution. Enantiomeric specificity, especially in transesterification reactions, allows production of specific chiral esters, which are important intermediates in production of pharmaceuticals and pesticides, as well as for the optical purification of racemic mixtures. Currently, none of the thermophilic or psychrophilic lipases are used in the production of chiral compounds at their respective temperatures.

V. Applications of Lipases

Lipases from bacterial and fungal origin are the most widely used in various biotechnological applications. In general, they are stable in wide range of organic solvents and many show high thermostability, they have wider pH and temperature optima range than lipases from eukaryotic origin, and they have a very diverse substrate range with high regio- and enantioselectivity making microbial lipases an important and attractive choice for many applications in organic synthesis. Lipases from mesophilic organisms occupy the vast majority of industrial applications, mainly because they were the first to be isolated, cloned, and characterized. Below follows an overview of the applications of lipases and their biotechnological importance (Balcão *et al.*, 1996; Bornscheuer *et al.*, 2002; Jaeger and Reetz, 1998; Jaeger *et al.*, 1994, 1999; Schmidt-Dannert, 1999; Villeneuve *et al.*, 2000).

A. Medical Biotechnology

Lipases are increasingly on demand for the nutraceuticals industry, that is, for substances that can be considered a food but that provide medical or health benefits, including the prevention and/or treatment of a disease. For example, the lipase from *Pseudomonas* sp. immobilized within the walls of a hollow-fiber reactor was used to change the linoleic acid present in corn oil into conjugated linoleic acid (CLA) which is a nutraceutical that has anticarcinogenic and antiatherogenic activities (Sehanputri and Hill, 2000).

Obesity is regarded recently as one of the top health issues in the industrialized world; for example, it is a serious medical disease that affects over a quarter of adults in the United States, and about 14% of children and adolescents according to America Obesity Association (AOA) (http://www.obesity.org/). One of the possible treatments for obesity is developing lipase inhibitors. Orlistat© is the first lipase inhibitor among antiobesity drugs; it has been shown to reduce body weight by inhibiting absorption (by ~30%) of ingested dietary fat and was proposed as a novel approach for obesity treatment (Lucas and Kaplan-Machlis, 2001).

Indomethacin, ketoprofen, and etodolac are nonsteroidal anti-inflammatory drugs (NSAIDs). Their therapeutic efficacy is often limited because of their poor aqueous solubility and permeability (Akimoto and Nagase, 2003). Forming sugar esters using lipases is effective in enhancing drug solubility, and is quite effective in preparing prodrugs; for example, Wang *et al.* (2005) used the lipase from the psychrophile *Candida antarctica* to catalyze the transesterification of glucose with vinyl esters of indomethacin, ketoprofen, and etodolac.

B. Detergent Industry

An estimated 1000 tons of lipases are added to ~13 billion tons of detergents produced each year (Godfry and West, 1996). Novo Nordisk, the leading company for industrial enzymes, introduced many lipases as detergent additives among other various industrial applications. They produced the first commercial thermophilic lipase named Lipolase® from *Thermomyces lanuginosus* and is was used in the detergent industry. Other bacterial lipases like Lumfast™ from *Pseudomonas mendocina* and Lipomax™ from *Pseudomonas alcaligenes* were produced from mesophiles by Genencor International and are used as detergent additives (Jaeger *et al.*, 1999). Although compared to recent discovered enzymes, the presently used enzymes are not the most efficient, thermostable, or

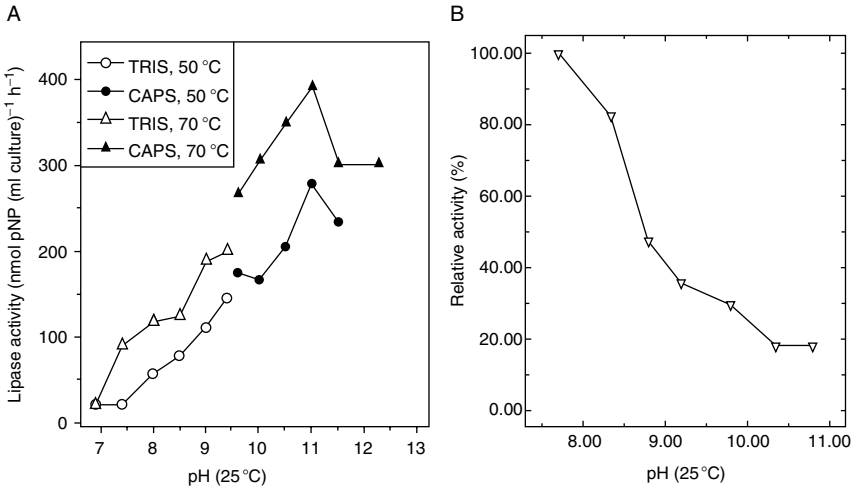

FIG. 5. The effect of pH on lipolytic activity of *Thermosyntropha lipolytica* lipases (A) and the recombinant Lipolase® (B) from Novo Nordisk (courtesy of V. Svetlitshnyi).

detergent resistant lipases (Fig. 5). However, in respect to the gain, apparently the industrial development and change of the production process is too costly to change the process at this time.

C. ORGANIC SYNTHESIS

Lipases in organic synthesis are primarily used to catalyze enantioselective reactions for the synthesis of fine chemicals and especially in preparing chiral intermediates for pharmaceuticals (Klibanov, 2001; Patel, 2000).

In the field of therapeutics, examples include lipases that are used in the synthesis process of macrolide products (e.g., epothilones), which exhibit potent antitumor activity against a wide spectrum of human tumor cell lines including multidrug resistant cell lines (Broadrup *et al.*, 2005). To introduce chirality at C_{15} position in the synthetic epothilone D (Broadrup *et al.*, 2005) and epothilone A (Zhu and Panek, 2000, 2001), a highly enantioselective lipase from *Pseudomonas* sp. was applied in the process. *Candida rugosa* lipase catalyzes the enzymatic resolution of the antimicrobial compounds (*S*)- and (*R*)-elvirol and their derivatives (*S*)-(+)- and (*R*)-(–)-curcuphenol (Ono *et al.*, 2001). The lipase Novozym 435 from *Candida antarctica* B was used to catalyze acylation of the immunosuppressant and antifungal agent rapamycin and 42-ester

derivatives (e.g., rapamycin 42-hemisuccinate, 42-hemiadipate) with various acylating agents with complete regioselectivity and high yields (Gu *et al.*, 2005), a psychrophilic lipase, LipB68, from *Pseudomonas fluorescens* B68, effectively catalyzed the transesterification of both α-phenylethanol and α-phenylpropanol at 20°C (Luo *et al.*, 2006). Again, an area where novel lipases from extremophiles such as low-temperature active enzymes for synthesis of temperature-sensitive compounds has a promising potential.

D. Biodiesel Production

Biodiesel, a monoalkyl fatty acid ester (preferentially methyl and ethyl esters), is presently evaluated as a replacement for diesel. Biodiesel has a few advantages over petroleum diesel; it is biodegradable; its combustion products have reduced levels of particulates, carbon oxides, and sulfur oxides (Iso *et al.*, 2001).

Lipases from mesophiles, psychrophiles, and thermophiles have been tested for the production of biodiesel including the enzymes from the mesophile *Rhizomucor miehei* (immobilized on ion-exchange resins) and *Thermomyces lanuginose*, a thermophilic lipase, immobilized on silica gel used for the conversion of sunflower oil into biodiesel by methanolysis (Al-Zuhair, 2005). Lipases from *Burkholderia cepacia* (Noureddini *et al.*, 2005) and Novozym 435 (Du *et al.*, 2004) were used to convert soybean oil. LipB68 from *Pseudomonas fluorescens* B68, a psychrophilic lipase, was found to be effective in esterifications and biodiesel production at 20°C (Luo *et al.*, 2006).

Several processes for biodiesel fuel production have been developed, among which transesterification using alkali-catalysis gives high levels of conversion of triglycerides to methyl esters in short reaction times. This process has, therefore, been used for biodiesel fuel production in a number of countries, including Belgium, France, Germany, Italy, and United States, with fuel production exceeding 100,000 tons yearly (Fukuda *et al.*, 2001). Alkali- and thermostable lipases/esterases from alkalithermophiles should have an advantage over those from mesophilic microorganisms. However, again the savings have to be greater than the cost for the development of a new industrial process. The LipA/B lipases from *Thermosyntropha lipolytica*, although active at alkaline pH and elevated temperature, it does not exhibit a significant activity in the esterification assay with ethanol or methanol (M.S., unpublished results). However, a thermophilic lipase from *Bacillus* sp. J33 was used to catalyze the synthesis of methyl oleate at 60°C, which could be successfully used for biodiesel production (Nawani *et al.*, 2004).

E. Agrochemical Industry

In the agrochemical industry, lipases have been used in the synthesis of herbicides. Indanofan is a novel herbicide used for grass weeds in paddy fields. It was commercialized as a racemic mixture in 1999; however, by examining the herbicidal activity of each enantiomer, only the (*S*)-enantiomer is active. To synthesize this enantiomer, a combination of lipase-catalyzed enzymatic resolution and chemical inversion techniques were successfully used (Tanaka *et al.*, 2002).

F. Flavor and Aroma Industry

Producing flavor esters by extraction from plant sources and fermentation is time-consuming, expensive, and restricted to the supply of natural materials. One alternative is the use of lipases to catalyze the production of flavor and fragrance (Claon and Akoh, 1993; González-Navarro and Braco, 1998; Karrachaabouni *et al.*, 1996). Among the several examples of lipases in the development of food flavor is the use of the *Mucor miehei* lipase to catalyze esterification of citronellol and geraniol with short-chain fatty acids (Laboret and Perraud, 1999). Fungal and pancreatic lipases were used to enhance lipolysis reactions and the development of piquant flavor and sharp odor in Idiazabal cheese (a cheese made from raw ewes' milk) (Barron *et al.*, 2004).

(–)-Menthol is a component of peppermint oil and is produced on industrial scale by optical resolution of (±)-menthol. (–)-Menthol and its esters are more important from the industrial point of view than (±)-menthol. (–)-Menthol, because of its cooling and refreshing effects, is an important fragrance and flavor compound that is used largely in cosmetics, toothpaste, chewing gums, cigarettes, sweets, and medicines. (–)-Menthol was synthesized by enantioselective transesterification of (±)-menthol using *Burkholderia cepacia* lipase (Athawale *et al.*, 2001).

G. Food Industry

Fats and oil modification is one of the presently investigated areas in food-processing industry, tailored vegetable oils with nutritionally important structured triacylglycerols and altered physicochemical properties could have a big market value. It is the main interest of members in the European Federation for the Science and Technology of Lipids (http://www.eurofedlipid.org/). Lipases, especially microbial lipases, which are regiospecific, are exploited for retailoring of vegetable oils

(Gupta *et al.*, 2003). A good example of processing fat is the use of immobilized lipases to catalyze batch-directed interesterification of tallow, resulting in oleins containing significantly higher levels of unsaturated fatty acids than obtained by fractionation without lipase (MacKenzie and Stevenson, 2000).

Several lipases are used to catalyze the synthesis of structured lipids. In order to produce one kind of reduced-calorie structured lipids, different lipases were used to catalyze the incorporation of short-chain fatty acids (acetic, propionic, and butyric acids) into triolein (Tsuzuki, 2005). *Thermosyntropha lipolytica* lipases LipA and Lip B were shown to effectively catalyze the synthesis of diacylglycerols, especially 1,3-dioleylglycerol, at 85°C (Salameh, 2006).

VI. Conclusions

Lipases are interesting enzymes which are found in almost every type of living organisms. Lipases constitute the most important group of biocatalysts for biotechnological applications. Structurally, they have conserved 3D structures but lack a high-sequence homology and display a wide diversity of properties. Commercially, they are used in multiple applications and because of this, the search for novel lipases continues. As is obvious from this chapter, little is known about lipases from extremophiles, especially derived from psychrophiles, true halophiles (no lipase characterized), acidophiles, and piezophiles (no lipase characterized). Characterized lipases from extremophiles are mainly from aerobic thermophiles. It is expected that due to the need for cold active lipases for the laundry detergent industry, more lipases active at below 20°C and alkaline pH from psychrophiles, including psychrophilic/tolerant alkaliphiles will be described. The more enzymes being discovered from organisms in extreme environments, the more exceptional features will be discovered. An example are the two lipases from the slightly alkalithermophile *Thermosyntropha lipolytica* (from Lake Bogoria, an alkaline lake with hot springs) exhibiting high thermostability and activity above 90°C and at alkaline pH. Thus, the authors encourage the interested reader to study lipases from organism living under any of the above extreme conditions. However, for industrial application, besides exhibiting the desired properties, these enzymes also have to have a high level of expression, that is, efficient overexpression of their genes in a suitable host. The special stability and activity ranges of lipases from extremophiles, different from most mesophilic host protein, offer easy and simple purification steps by employing those extreme conditions.

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In Situ Bioremediation

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I. Introduction

In situ bioremediation is the biological treatment of contaminated soil and groundwater without excavating the soil or without pumping and treating groundwater above soil. It relies on the use of microbes or plants to degrade or immobilize contaminants *in situ* and the technology brings together a combination of microbiological, chemical, geological, and engineering sciences (Table I). More than 1000 patents have been granted to different technical *in situ* bioremediation aspects. The location of contaminants in soil and groundwater is the results of the man-made actions that have caused the contamination. Topsoil may be contaminated due to airborne pollution, unintended surface soil or shoreline spills of chemicals, or intended deposition of contaminants on the surface soil. In most cases, the contamination, however, is not limited to the surface soil, but extends or appears in the deeper soil layers and in the groundwater, due to leaching from topsoil or spills from underground storage tanks or pipes or buried wastes. Since surface soil is easily accessible and can be handled using, for example, agricultural practices, numerous bioremediation cases have been performed on topsoil or excavated soil with good success. The treatment of deeper layers of soil and groundwater is a bigger challenge. This chapter focuses on field applications of *in situ* treatment of deeper layers of soil and groundwater.

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TABLE I

In Situ Bioremediation Principles and Technologies

Target zone	Principle	Engineered remedial action	Bioaugmentation	Monitoring
Unsaturated zone				
Topsoil	Natural attenuation	*Monitored natural attenuation*: No technical actions	–	Soil
Topsoil	Enhanced natural attenuation	*Landfarming*: Nutrient addition (granulated or dissolved)	+/–	Soil
		Phytoremediation: Plant and nutrient addition	+/–	Soil Rhizosphere Plants
Subsurface	Natural attenuation	*Monitored natural attenuation*: No technical actions	–	Soil Soil gas
Subsurface	Enhanced natural attenuation	*Bioventing*: Oxygen addition through injection wells	+/–	Soil Soil gas
		Nutrient infiltration: Injection of nutrients dissolved in water	+/–	Soil Soil gas
Saturated zone	Natural attenuation	*Monitored natural attenuation*: No technical actions	–	Soil/sediment Groundwater
	Enhanced aerobic natural attenuation	*Air sparging*: Oxygen gas supply through injection wells	+/–	Soil/sediment Groundwater
		Oxygen release: Oxygen released from slurried MgO_2 though injection wells	+/–	Soil/sediment Groundwater
	Enhanced anaerobic natural attenuation	*Electron acceptor addition*: Injection of dissolved nitrate or sulfate	+/–	Soil/sediment Groundwater
		Electron donor addition: Injection of lactate, methanol, and so on, in solution	+/–	Soil/sediment Groundwater

II. Unsaturated Zone Treatment Methods

The unsaturated zone comprises the soil layers above the groundwater level and is also termed the *vadose* zone. The *in situ* bioremediation of this zone relies mainly on aerobic biodegradation of contaminants or on the enhancement of aerobic degradation (Table I), but particularly in cases where the contaminant source is below ground, the natural microbial activity will rapidly consume all oxygen and the unsaturated zone will become anoxic. The natural degradation of contaminants without human intervention is called *natural attenuation*. This term can cover both the unsaturated and saturated zones and thus the remediation of both soil and groundwater. The use of monitored natural attenuation (MNA) as a remediation technology for groundwater is further described in Section III.A.

A. Natural Attenuation

The natural biodegradation of contaminants in surface and subsurface unsaturated soils is most directly shown by the true disappearance of the contaminants by the chemical analysis in soil samples over time (Jørgensen *et al.*, 2005a), and this is what the authorities usually require. It is, however, difficult to obtain representative samples for repeated sampling over time, since soil is heterogeneous and soil samples from a certain point and depth can only be taken once. Consequently, time series will always consist of samples from different drilling or digging pits. Since contaminants are unevenly distributed in soil, this gives an extra challenge for proving successful *in situ* bioremediation. For this reason, the *in situ* biodegradation rates are often estimated based on oxygen consumption or carbon dioxide production based on a stoichiometric calculation of, for example, petroleum hydrocarbon (CH_2) conversion to carbon dioxide and water:

$$CH_2 + 1.5O_2 \rightarrow CO_2 + H_2O$$

From this equation, it can be calculated that 1.5-mol O_2 per mol CH_2 or equivalently 3.4-mg O_2 g^{-1} of CH_2 is needed for the complete degradation of petroleum hydrocarbons. Due to the microbial respiration of other organic material in soil, these kinds of calculations are not very accurate. Also the ignorance of formed microbial biomass (empirical formula $C_5H_7O_2N$) may cause biases. Despite these shortcomings, the use of this formula to model bioremediation of petroleum hydrocarbons has proven useful.

In passive bioremediation techniques, the oxygen availability is controlled by the diffusion of oxygen from the overlying air through

the soil body. Huesemann and Truex (1996) found that oxygen is expected to penetrate most contaminated soil for up to several meters if hydrocarbon biodegradation rates are similar to those measured during bioventing tests, which is typically 2.5 to 10 mg of total petroleum hydrocarbon (TPH) kg^{-1} day^{-1}.

There is a lot of interest in low-temperature bioremediation since many petroleum hydrocarbon spills have taken place also in the arctic. Rike *et al.* (2003) investigated the *in situ* biodegradation in frozen arctic soils by assessing the oxygen and carbon dioxide consumption and production. The petroleum hydrocarbon degradation rate was modeled based on the microbial oxygen consumption rate and the bulk density of the soil assuming that hydrocarbons (CH_2) were degraded into carbon dioxide and water without the formation of biomass. They were able to model hydrocarbon degradation rates in the range of 3.0 to 7.1 mg TPH kg $soil^{-1}$ day^{-1} at temperatures at or below 0°C. They suggested that bioremediation may proceed for certain period during the winter. In another study in an Alpine Glacier skiing area, Margesin and Schinner (2001) found that hydrocarbon degradation in lysimeters without any biostimulation stimulation was in the order of 2.1 mg TPH kg^{-1} day^{-1}.

Recently, more attention has been paid to the natural development of anoxic conditions in the unsaturated subsurface when the contamination source is located just above the groundwater level. This is often the case with petroleum hydrocarbons, which typically float on water and spread around the *capillary fringe*. Bekins *et al.* (1999) found at the anoxic core of a crude oil plume at the Bemidji site in Minnesota that the unsaturated zone contained higher numbers of methanogens than the saturated zone. They found that iron reduction, fermentation, and methanogenesis were the dominating microbial process also in the unsaturated zone. In that study, the actual petroleum hydrocarbon degradation rates in the unsaturated zone were not assessed. Salminen *et al.* (2004) investigated the unsaturated zone of a former landfill site in Finland where oily waste had been dumped. They found that the unsaturated zone was anoxic with methane and carbon dioxide concentrations in the soil gas up to 25% and 18% (v/v), respectively. A high potential for both aerobic and anaerobic removal of the indigenous petroleum hydrocarbons in the range C_{10}—C_{40} was documented in unamended microcosms at 7°C, which represent the near *in situ* temperature. Furthermore, they showed that indigenous alkanes were degraded under methanogenic conditions. This is one of the first studies to show that anaerobic alkane degradation really seems to be of significance in the nature (Table II). At this site, the dominating

TABLE II

STUDIES OF ALKANE DEGRADATION UNDER METHANOGENIC CONDITIONS AT PETROLEUM HYDROCARBON-CONTAMINATED SITES UNDERGOING NATURAL ATTENUATION

Site and contamination	Method	Incubation temperature (°C)	References
Bemidji, Minnesota; Crude oil, pipeline spill	Amendment with ^{14}C-hexadecane to aquifer sediment	20	Anderson and Lovley, 2000b
	Degradation patterns of alkanes in the plume with prevailing methanogenesis	No incubation	Bekins *et al.*, 2005
Trollberget, Finland	No amendment	7	Salminen *et al.*, 2004
Landfilled light weight waste oil	Disappearance of contaminants present in soil and aquifer sediment		
Mildred Lake, Alberta; Oil sand tailings + alkane containing waste diluent	Amendment with *n*-alkanes (C_6–C_{10}) to a slurry of mature fine tailings	22	Siddique *et al.*, 2006
Shiziuoka, Japan; Refinery crude oil	Amendment with decane to a soil slurry from the capillary fringe	25	Kasai *et al.*, 2005
Ft. Lupton, Colorado; Natural gas field site	Amendment of crude oil to aquifer sediment slurries	Room temperature	Townsend *et al.*, 2003

anaerobic processes were also iron reduction, fermentation, and methanogenesis (Salminen, 2005; Salminen *et al.*, 2006). Furthermore, Tuomi *et al.* (2004) studied the aerobic complete degradation potential of naphthalene well as the gene copy number of the *nahAc* gene, which encodes for naphthalene dioxygenase, in depth profiles at the same site. It was found that aerobic naphthalene complete degradation potential and *nahAc* genes were present in the anoxic parts of the unsaturated zone, but they were correlated with each other only in the aerobic zone. The use of molecular techniques for the monitoring of bioremediation is further discussed in Section V.

Kasai *et al.* (2005) investigated the capillary fringe at a crude oil contaminated site in Japan and they found that both anaerobic and aerobic BTEX (benzene, toluene, ethylbenzene, and xylene) and decane degradation potential were present in amended soil slurries at 25°C. They also investigated the microbial community by cloning and sequencing of amplified bacterial and archaeal 16 sRNA genes fragments from soil samples. They found that the dominating groups of bacteria were composed of *β*-, *γ*-, *δ*-, *ε*-*proteobacteria*, and *Acidobacteria*. Also *Spirochaeta*, *Actinobacteria*, *Bacteroidetes*, and *Nitrospirae* were represented. Several clones were closely related to known aerobic and anaerobic hydrocarbon degraders (sulfate reducers and fermenters) and methanotrophs. Among archaeal clones, close relatives to *Methanosaeta* were detected indicating the capacity for *aceticlastic methanogenesis*, which is the formation of methane and carbon dioxide from acetate.

These studies of natural attenuation in the unsaturated zone all show that aerobic potential is present in the anoxic part of the plumes. Methanotrophs may frequently be found. This emphasizes the vast microbial diversity of the unsaturated zone, and it seems that aerobic and anaerobic metabolisms function in close vicinity of each other. Iron reduction, fermentation, and methanogenesis seem to be the dominating anaerobic processes at several sites.

B. Enhanced Natural Attenuation

Aerobic biodegradation in the unsaturated zone can be enhanced by pumping air or pure oxygen into the subsurface through gas injection wells installed into the soil. This technology is called *bioventing*. It can be combined with the extraction of volatile contaminants like BTEX or chlorinated solvents from the soil gas phase. Furthermore, the natural attenuation can be enhanced by nutrient addition.

1. *Bioventing*

Bioventing was one of the first technologies to be applied in large scale in the 1990s (Leeson and Hinchee, 1997) and is now widely used in commercial applications. The installation of injection wells allows for the performance of an *in situ respiration test* in which the oxygen consumption in the soil gas is determined after aerating the soil and then closing the air pumping. Leeson and Hincee suggested that respiration rates of more than 1% per day is a good indicator that bioventing may be feasible at a site. Bioventing can be performed as an active or passive technology. In *passive bioventing*, only the atmospheric pressure affects on the gas exchange from the *vent wells*, whereas in active bioventing, air is forced into the ground by a blower possibly in connection with a vacuum extraction of the gas (Zimmerman *et al.*, 2003).

Active bioventing was successfully applied at two petroleum hydrocarbon-contaminated sites in Austria containing mainly nonaromatic hydrocarbons (Aichberger *et al.*, 2005). The bioventing process was followed by respiration tests over time, which showed decreasing rates of respiration. At one site, the oxygen consumption rate was 9% per day (at 7000 mg TPH kg^{-1}) after 1 year of operation, and decreased gradually over the next 4 years till below 1% per day when concentrations of TPH reached 500 mg TPH kg^{-1}. At the other site, the respiration rate decreased more than 10 times over the first 2 months. At this site, the contaminant concentrations varied from 2000 to 20,000 mg TPH kg^{-1} and respiration rates were measured at 13 different points. The respiration rates varied between 13.1% and 2.2% per day at the initiation of the bioventing, and varied between 1.2% and 0.4% per day after 3 months. The authors highlighted that the test is describing the respiration only at one point at one time even if it is corrected for the natural respiration background from a clean point nearby. Furthermore, residual concentrations of the contaminant cannot be estimated from respiration tests but progress soil boring had to be performed.

A large-scale bioventing and air sparging remediation was performed at a site in California, where both the unsaturated and saturated zones were contaminated with diesel and gasoline. Respiration tests showed the highest oxygen utilization rate of 5.3% per day. On the basis of results from respiration tests and the concentration of volatile organic compounds (VOC) in the exhaust air, it was calculated that more than 95% of the volatile hydrocarbons were biodegraded in comparison to vapors directly extracted. Progress soil borings confirmed the progress of the remediation, which was running for 6 years, and concentrations decreased from maximum 2300 to 100 mg kg^{-1} TPH as gasoline in the unsaturated zone (Corey and Stewart, 2003).

2. *Infiltration with Nutrients*

The main break through for bioremediation was the treatment of the Exxon Valdez crude oil spill on intertidal sediments in Alaska 1989 (Bragg *et al.*, 1994). They found a significant stimulating effect of nutrient addition, and they furthermore showed that the rate of biodegradation was a function of the nitrogen concentrations maintained in the porewater of the intertidal sediment. Even though this intertidal sediment does not represent the unsaturated zone, it represents a system where a direct surface addition of nutrients was performed. Most frequently, aerobic degradation is enhanced by the addition of nutrients. In many cases, the effect of nutrient additions alone have not been tested in field scale, but rather in laboratory preliminary tests together with aeration or inoculum addition (Aichberger *et al.*, 2005; Delille *et al.*, 2002; Gogoi *et al.*, 2003; Katsivela *et al.*, 2005; Margesin and Schinner, 2001).

III. Saturated Zone Treatment Methods

The saturated zone is the zone below the groundwater table. Pollution of this zone from a point source either occurs through the percolation through the unsaturated zone or through a direct leakage, for example from underground storage tanks or dumpsites to these deeper layers either dissolved in water or as a free phase of *nonaqueous phase liquids* (NAPLs). It is evident that water soluble compounds may spread more rapid with the groundwater than NAPLs. However, *light nonaqueous phase liquids* (LNAPLs) like oil hydrocarbons that are lighter than water may float on top of the groundwater level and spread along the groundwater surface or get stuck in the capillary fringe. In contrast, *dense nonaqueous phase liquids* (DNAPLs) such as chlorinated solvents that are heavier or more dense than water may move down through the groundwater column and spread along the bottom of the groundwater zone, for example along the bedrock surface. In the saturated zone, oxygen is dissolved in water and therefore the availability of oxygen is limited in contrast to the availability in soil air. Remediation technologies for the saturated zones are therefore focused on the manipulation of the groundwater (Table I).

A. Natural Attenuation

MNA is the monitoring of natural processes in soil and groundwater environments that act *without human intervention* to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in those media (EPA, 1999). When using natural attenuation as a remediation

strategy, the total destruction of the contaminant, for example, by biodegradation is preferred compared to, for example, dilution. Biodegradable contaminants such as petroleum hydrocarbons are thus best suited for natural attenuation. Natural biodegradation may take time, but viewed on a long-term scale it may be more sustainable than many other remediation technologies, since no excavation or pumping of groundwater is performed, and neither transport nor final deposition place for the contaminated soil is needed. The main cost for MNA is the performance of a proper site investigation and risk assessment, which has to be performed anyway, as well as long-term monitoring and handling of monitoring data.

Natural attenuation involves anaerobic degradation of the contaminants in the cases where oxygen is used up near the core of the contaminant plume. A sequence of anaerobic processes develops around the core of the plume and indicates indirectly that the contaminant is biodegraded. These anaerobic processes progress according to their differences in oxidation–reduction potentials in the order: denitrification, manganese reduction, iron reduction, sulfate reduction, and fermentation followed by methanogenesis. Often one or more anaerobic processes become dominant. The EPA (1999) of the United States was the first to propose that three lines of evidence for natural attenuation should be applied to demonstrate that natural attenuation is taking place: (i) documented decrease in contaminant concentrations at the site, (ii) documented geochemical or geomicrobiological conditions resulting from biological activities, and (iii) documented microbiological degradation activity. These principles have been adopted by many European countries as well (Rügner *et al.*, 2006). These principles have fostered a lot of emphasis on the groundwater chemistry in order to demonstrate (i) and (ii), as well as on laboratory microcosms to demonstrate (iii). Particularly the evidence for criterion (i) has enhanced the modeling of contaminant migration. Mulligan and Yong (2004) reviewed models available for the modeling of natural attenuation. The existing models are mainly concerned with groundwater and there is a need for integration of soil and groundwater models. Many models take the biodegradation rate into account, but one major problem is the lack of biodegradation rates obtained from field studies and the heterogeneity of the sites. Furthermore, Maurer and Rittmann (2004) emphasized that there is a need to include fermentation and abiotic chemical processes into the available computer models. The evidence for criterion (ii) is often given based on a battery of groundwater analysis that reveal the progress of anaerobic processes, with parameters like dissolved oxygen, nitrate, iron (II), sulfate, methane,

and dissolved inorganic carbon (DIC) such as bicarbonate (Bolliger *et al.*, 2000). Furthermore, stable isotope analysis of the organic and inorganic carbon can be used to evaluate the *in situ* turnover of contaminants. Differences in isotopic fractionation over time (change of isotopic ratio ($\delta^{13}C$) due to preferential use of one of the involved isotopes) of the source and the DIC can be used to evaluate whether the DIC is originating from the contaminant, and is thus an evidence for the biodegradation (Grossman, 1997).

Numerous field studies have shown that natural attenuation of contaminants is indeed taking place in the saturated zone as reviewed by Scow and Hicks (2005). The most frequent compound groups that have been evaluated are BTEX compounds, TPH, the gasoline oxygenate methyl *tert*-butyl ether (MTBE), and chlorinated solvents. Most success has been observed for BTEX and TPH, whereas both MTBE and chlorinated solvents seem to need enhanced remediation. The degradation of petroleum hydrocarbons under anaerobic conditions has largely been underestimated in earlier literature and for long time even text books would state that this is not possible. During the late 1980s and 1990s, evidence was created on the anaerobic degradation of aromatic hydrocarbons, which have been reported to be degraded under denitrifying, iron-reducing, sulfate-reducing, and fermentative and methanogenic conditions as reviewed by Chakraborty and Coates (2004). Later also evidence for methane formation from long-chain alkanes has appeared (Zengler *et al.*, 1999). This ''alkane cracking'' is suggested to happen in a consortium of syntrophic bacteria and methanogenic archaea:

$$4C_{16}H_{34} + 64H_2O \rightarrow 32CH_3COO^- + 32H^+ + 68H_2$$

$$32CH_3COO^- + 32H^+ \rightarrow 32CH_4 + 32CO_2$$

$$68H_2 + 17CO_2 \rightarrow 17CH_4 + 34H_2O$$

Net reaction:

$$4C_{16}H_{34} + 30H_2O \rightarrow 49CH_4 + 15CO_2$$

Members of the consortium carrying out these reactions were identified by sequencing of clones of 16 sRNA genes directly retrieved from an enrichment culture, and they were closely related to *Syntrophus* spp., *Desulfovibrio* sp., *Methanosaeta* sp., *Methanospirillum*, and *Methanoculleus.* Several field studies (Table II) have later verified that alkane degradation is taking place in anaerobic samples from contaminated sites (Anderson and Lovley, 2000b; Bekins *et al.*, 2005; Salminen *et al.*, 2004; Siddique *et al.*, 2006). It can be concluded that a consortium of fermentative bacteria is very important in the first part of the

degradation of petroleum hydrocarbons, and it has been found that the initial attack on the alkane or substituted monoaromate is fumarate addition as reviewed by Widdel and Rabus (2001). Since the anaerobic degradation of petroleum hydrocarbons often is a result of a microbial consortium, it is interesting to see which organisms are present in petroleum hydrocarbon plumes undergoing natural attenuation.

Dojka *et al.* (1998) investigated the microbial diversity in a hydrocarbon and chlorinated solvent contaminated aquifer in Michigan undergoing natural attenuation by using culture-independent methods. They found a vast diversity of bacteria and archaea in groundwater, and in the methanogenic zone of the aquifer they found two particular abundant sequence types, one being *Syntrophus* spp., known to produce energy from the anaerobic oxidation of organic acids, with the production of acetate and hydrogen. The other frequent sequence type was closely related to *Methanosaeta* spp., which produces methane and carbon dioxide from acetate. They proposed that the terminal step in hydrocarbon degradation in the methanogenic zone of the aquifer was aceticlastic methanogenesis and that the organisms would occur in syntrophic association. The dominance of *Methanosaeta* indicating dominance of aceticlastic methanogensis was also found in a heating oil-contaminated Swiss aquifer (Bolliger *et al.*, 2000; Kleikemper *et al.*, 2005), but these authors suggested that both aceticlastic and CO_2-type substrate consuming methanogens are likely to be involved in the terminal step of the hydrocarbon degradation. von Wintzingerode *et al.* (1999) found in a study of a dechlorinating fluidized bed reactor several clones closely affiliated with the sequences of uncultured clones found in the study by Dojka *et al.* (1998). Important groups of organisms in this study were, for example, *Spirochaeta*, *Syntrophus*, and many other groups related to known fermenters. They also found that clone sequences closely related to *Methanosaeta* were dominating. Most of these studies have been based on clone libraries made from groundwater samples, and it would be interesting to investigate samples that contain the aquifer sediment to see whether similar sequences dominate. Bekins *et al.* (1999) found that only about 15% of the total microbes were present in the water phase and 85% were attached to the sediment particles in a contaminated aquifer.

B. Enhanced Aerobic Natural Attenuation

The aerobic natural attenuation of contaminants in the saturated zone can be enhanced by adding oxygen. This can be done by *air sparging* in which air or pure oxygen is pumped directly into the

groundwater or by injecting aerated water to the plume through installed perforated injection wells (Knapp and Faison, 1997). The fact that oxygen is not very soluble in water, large amounts of injected water would be needed and this causes displacement of the contaminated groundwater. Injection of water is mainly used if the other cosubstrates such as nutrients or inocula have to be added at the same time. The injection of oxygen may also enhance chemical oxidation reactions, for example iron oxidation, and thus the technology is not very efficient (Shields *et al.*, 2003). The most successful oxygen injections have been performed in the context of MTBE bioremediation with or without simultaneous inoculation. The addition of an aerated MTBE-degrading culture (Table III) was done by feeding the groundwater continuously and an intermittent direct oxygen injection into the groundwater was applied (Salanitro *et al.*, 2000). They noted that increased gas saturations may cause altered hydraulic properties. The oxygen levels increased in the groundwater and MTBE degradation was observed in this pilot scale field study. Another study where no addition of inoculum was done showed efficient MTBE degradation in a system where oxygen was added by diffusion through the walls of oxygen pressurized polymeric tubes placed in contact with the flowing

TABLE III

EXAMPLES OF SUCCESSFUL BIOAUGMENTATIONS IN FULL SCALE

Target compound	Inoculum	References
MTBE[a]	*Methylobium petroleophilum* PM-1	Smith *et al.*, 2005; Scow and Hicks, 2005
MTBE	Mixed culture MC-100	Salanitro *et al.*, 2000
Carbon tetrachloride	*Pseudomonas stutzeri* KC	Dybas *et al.*, 1998; ESTCP, 2005
TCE[b], PCE[c]	Mixed culture KB-1 containing relatives to *Dehalococcoides* sp.	Major *et al.*, 2002; ASTCP, 2005
TCE, PCE	Mixed culture Pinellas culture containing relatives to *Dehalococcoides* sp.	Ellis *et al.*, 2000; ESTCP, 2005
TCE, DCE, VC[d]	*Burkholderia cepacia* ENV435	Steffan *et al.*, 1999; ESTCP, 2005

[a]Methyl *tert*-butyl ether.
[b]Trichloroethene.
[c]Tetrachloroethene.
[d]Vinyl chloride.

groundwater (Wilson *et al.*, 2002). Oxygen can also be introduced by electrolysis or by injection of slurries of solid forms of oxygen such as magnesium/oxygen compounds (Table I), which are commercially available (Wilson, 2003).

C. Enhanced Anaerobic Natural Attenuation

The fact that oxygen as a gas has a limited solubility in groundwater and that anaerobic processes start to dominate in the subsurface after a spill has led to new perspectives of adding alternative electron acceptors such as nitrate or sulfate to the groundwater. These compounds are very soluble in water. The main concerns for the safe use of electron acceptor additions are from the authorities' side the possible accumulation of, for example, nitrate and nitrite or sulfide, which may cause toxicity, odor, or taste problems in the water used for drinking purposes.

Nitrate addition was tested at a diesel oil-contaminated site in Switzerland (Hunkeler *et al.*, 1999). The nitrate was consumed very fast and no excessive production of ammonium, nitrite, or nitrous oxide was observed. It was concluded that the nitrate was reduced to free nitrogen in a complete denitrification process. There was, however, a larger production of inorganic carbon than was calculated based on the stable isotope balances, and it was concluded that the complete degradation of petroleum hydrocarbons may be underestimated, if based only on the oxidant disappearance.

Sulfate addition in order to enhance sulfate reduction was tested at a refinery site in Oklahoma (Anderson and Lovley, 2000a) and the degradation of benzene in the groundwater was followed. The benzene concentrations decreased along the groundwater flow path. It was assumed that produced sulfide most likely precipitated with ferrous iron, which was naturally present in the aquifer.

Perhaps the most successful manipulation of environmental conditions for the enhanced anaerobic natural attenuation is the addition of electron donor to create reduced conditions in order to stimulate reductive dechlorination of chlorinated solvents. Hydrogen may be directly injected into an aquifer (Aziz *et al.*, 2003), or through the addition of electron donor in the form of a fermentable carbon source such as lactate (Ellis *et al.*, 2000; Romer *et al.*, 2003), fumarate (Hageman *et al.*, 2004), or methanol (Dyer *et al.*, 2003), which on fermentation is converted into hydrogen, which is used in the dechlorination process. Many of these applications have been successful and are today used in large scale often together with addition of inocula for the dechlorination.

IV. Use of Inocula

The use of inocula or *bioaugmentation* by pure or mixed cultures of microbes has been the goal of many development projects for efficient *in situ* bioremediation technology, but so far there are not so many success stories in full scale. The reason for this is the lack of the joint understanding of the chemistry, geology, and microbial ecology of the contaminated subsurface. Furthermore, the technical solution for the inoculation into this kind of heterogeneous system is a great challenge as well as the monitoring of the progress of the bioremediation. Thompson *et al.* (2005) suggested that selection for strains should not be solely based on the degradation ability but rather on the understanding of the environment where it is supposed to function. It has, however, been observed that differences in degradation abilities may vary between strains of the same bacterium. It has been shown that all known cultures that dechlorinate trichloroethene (TCE) and tetrachloroethene (PCE) beyond *cis*-dichloroethene (DCE) contain organisms belonging to the genus *Dehalococcoides*, but not all *Dehalococcoides* stains dehalogenate chloroethenes (ESTCP, 2005).

The most successful bioaugmentation full-scale cases, which are listed in Table III, have been made with MTBE degraders (Salanitro *et al.*, 2000; Stocking *et al.*, 2000) and dechlorinators (Major *et al.*, 2002; Steffan *et al.*, 1999; Sleep *et al.*, 2006) as also concluded in the review by Scow and Hicks (2005). In contrast, petroleum hydrocarbon degraders are very easily enriched *in situ* both under aerobic and anaerobic conditions, and very often the control without inoculation shows similar degradation rates with the noninoculated, however, with some exceptions (Mishra *et al.*, 2001). Bioaugmented bacteria are a subject to predation by protozoa, and attempts to increase the survival by encapsulating the inoculum, for example by gellan gum, have been performed (Moslemy *et al.*, 2002).

There is a lack of well-documented case studies, and to overcome this problem, the Environmental Security Technological Certification Programme of the US Department of Defense commissioned a report on the status of the bioaugmentation in order to enhance the safe use of inocula for reductive dechlorination (ESTCP, 2005).

V. Monitoring Methods

A big challenge in the successful use of *in situ* bioremediation is the monitoring of the progress *in situ* and preliminary feasibility studies. Due to the heterogeneity prevailing at contaminated sites, the

preliminary site investigation is of most importance. For *in situ* bioremediation not only the geological structure and spatial contaminant distribution is of interest but also the microbial community and the prevailing aerobic or anaerobic metabolism type. The development in molecular microbiology has provided methods such as *cloning and sequencing* of amplified 16 sRNAgenes by which the microbial community composition can be assessed without isolation of pure cultures (Dojka *et al.*, 1998) as described earlier. The development of *microarrays*, *quantitative real-time PCR*, and better extraction methods for mRNA offers possibilities to quantify certain organisms or catabolic genes in environmental samples without long incubations for activity measurements. The genes involved in aerobic degradation of alkanes and mono- and polyaromatic hydrocarbons have successfully been quantified by hybridization and replicate limiting dilution PCR before real-time PCR became common, and were used to show a potential for degradation (Sanseverino *et al.*, 1993; Stapleton and Sayler, 1998; Stapleton *et al.*, 2000; Tuomi *et al.*, 2004). Rhee *et al.* (2004) did a pioneering work in developing microarrays for the detection of more than 2000 known degradation genes in the same assay using 50-mer oligonucleotides. However, the vast majority were aerobic genes and there is still a need to increase the sensitivity of the hybridization techniques. Genes involved in anaerobic degradation have been assessed by real-time quantitative PCR by Beller *et al.* (2002), who quantified the benzylsuccinate synthase gene *bss*, which encodes for the enzyme catalyzing the first step of fumarate addition to toluene. The anaerobic benzoyl coenzyme A reductase (BCR) genes, which encode the enzyme for the central step in the anaerobic degradative pathways for a variety of aromatic compounds, were assessed by Hosoda *et al.* (2005) by competitive PCR.

Molecular tools can also be used for the direct detection of inocula. An MTBE degrader (Smith *et al.*, 2005) and a dehalogenating inoculum (Major *et al.*, 2002; Sleep *et al.*, 2006) were detected by real-time PCR during bioremediation, and this technique is fast and promising also for more standardized assays. The presence of functional groups of bacteria such as iron reducers at sites undergoing natural attenuation has also been quantified by real-time PCR (Salminen *et al.*, 2006).

The presence of degradation products is another new challenging way of monitoring the progress of *in situ* bioremediation. For example, the formation of carboxylic acids formed during aerobic or anaerobic degradation of hydrocarbons is of interest. Gieg and Suflita (2002) were able to show the formation of several succinate derivatives of *n*-alkanes, cyclic alkanes, and alkylaromatic hydrocarbons from

hydrocarbon-contaminated groundwaters. The formation of organic acids during aerobic (Watson *et al.*, 2002) and anaerobic degradation of hydrocarbons as well as the formation of CO_2 which converts to HCO_3^- in groundwater cause an increase in the conductivity of the groundwater. This can be observed by using geophysical methods such as *electrical resistivity*, which can be performed as sounding from the soil surface without direct soil or groundwater sampling (Atekwana *et al.*, 2005; Jørgensen *et al.*, 2005b).

VI. Conclusions and Future Prospects

Progress in the full-scale use of *in situ* bioremediation has occurred mainly due the understanding of the ecology of the soil and groundwater system. The acknowledgment of the prevailing environmental conditions and development of, for example, anaerobic remediation technologies based on the new knowledge on the anaerobic degradation of petroleum hydrocarbons have created new possibilities for *in situ* bioremediation. The use of inocula has finally proven to be successful for the remediation of MTBE and chlorinated solvents. Furthermore, culture-independent methods for the identification of members of microbial consortia have provided new insight in the microbial ecology of contaminated sites. The expanding knowledge based on the sequencing of uncultured microorganisms and genomic research provides a basis for the development of more and better bioremediation strategies and inocula.

The inexpensive prices for dumping of excavated soil may diminish the incentive for bioremediation companies to invest into more demanding *in situ* biological systems. However, *in situ* remediation can be considered as a very sustainable technology, which does not require transport and deposition of contaminated soil. The collaboration between microbiologists, chemists, geologists, and engineers is of most importance for the development viable technologies. *In situ* bioremediation of the unsaturated zone is well established, and more challenges are linked to the *in situ* bioremediation of the saturated zone. More well-documented full-scale cases and technology evaluation schemes can help to facilitate the wider use of *in situ* bioremediation.

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Bacterial Cycling of Methyl Halides

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I. Introduction

The monohalomethanes methyl chloride (or chloromethane; MeCl), methyl bromide (or bromomethane; MeBr), and methyl iodide (or iodomethane; MeI) have received significant attention over recent years. Due to its high ozone-depleting potential, the use of MeBr in industrial and agricultural applications, for example, soil fumigation, is now being phased out under the Montreal Protocol (Sarma and Bankobeza, 2000). Concerns about the damaging effects of methyl halides have sparked investigations into the sources and sinks of these compounds. This has led to a better appreciation of the natural cycle of methyl halides. Degradation of methyl halides by microorganisms is recognized as a major sink for these compounds in the environment and a number of novel microorganisms involved in methyl halide degradation have been isolated. Studies of the metabolic pathways and the biochemistry and genetics of bacterial methyl halide degradation have given some insight into this important biological activity and some aspects of bacterial methyl halide degradation have been discussed in previous reviews (McDonald *et al.*, 2002; Trotsenko and Doronina, 2003). This chapter focuses on the monohalogenated methanes MeCl and MeBr, their natural and anthropogenic sources, and their degradation by microorganisms, specifically by aerobic bacteria that can use MeBr and MeCl as sole source of carbon and energy. The biogeochemical cycle of methyl halides, and the microbiology, biochemistry, genetics, and biotechnological potential of methyl halide-degrading microorganisms are discussed.

A. Role of Methyl Halides in Atmospheric Chemistry and as Ozone-Depleting Compounds

Methyl halides are the dominant halocarbons in the atmosphere. As such, they play an important role in regulating stratospheric ozone concentrations and to some extent, global warming as well, two factors governing planetary habitability. The monohalomethanes considered here, methyl chloride (MeCl), methyl bromide (MeBr), and methyl iodide (MeI), are trace gases in the atmosphere with average tropospheric-mixing ratios of 600, 10, and 2 parts per trillion (ppt), respectively (Table I). However, methyl halides are radiatively active and hence contribute to global warming by absorbing radiation in the infrared region. This is evident in their elevated global warming potential (GWP; Table I), a value calculated to quantify each compound's warming effect (on a mass basis)

TABLE I

PROPERTIES OF METHYL HALIDES AND THEIR EFFECT ON ATMOSPHERIC CHEMISTRY

Property	CH_3Cl	CH_3Br	CH_3I
Mixing ratio (ppt)	550	10	2
Lifetime (years)	1.3	0.7	0.02
Global warming potential[a]	17	5	N.A.
Ozone depletion potential	0.02	0.38	N.A.

[a]100-year time horizon.
N.A., not applicable.

relative to the same mass of CO_2. Compounds with high GWP, including those like methyl halides with low concentrations, may have considerable impact on atmospheric warming when compared with other ''greenhouse'' gases with low GWP.

More importantly, methyl halides are the major natural sources of halogen-containing radicals to the stratosphere (altitude >15 km) where they participate in the catalytic destruction of ozone. These reactions contribute to the ''ozone holes'' which have appeared during springtime in the upper atmosphere above polar regions since the 1980s (Solomon, 1999). Methyl halides are also partly responsible for the gradual thinning of the effective layer of ozone worldwide, leading to greater penetration of ultraviolet (particularly UV-B) radiation. It is estimated that MeBr contributes more than half of the Br present in the stratosphere (Cox *et al.*, 2005; Orlando, 2003) and that MeCl is responsible for at least 15% of chlorine-catalyzed ozone consumption (Montzka and Fraser, 2003). This is reflected in their elevated ozone depletion potential (ODP; Table I) which is calculated to indicate their ability to destroy ozone in the stratosphere, again on a mass basis relative to the same mass of the chlorofluorocarbon CFC-11 (CCl_3F).

Ozone-depleting compounds, including CFCs and MeBr, have been regulated since 1987 by the Montreal Protocol. This international agreement encourages signatory nations to decrease emissions of all ozone depleting compounds and has effectively reduced the burden of chlorine and bromine in the atmosphere. CFCs, halons (brominated compounds used as fire retardants), and other synthetic compounds were the first to

be phased out and their concentrations in the atmosphere are decreasing (Hurst *et al.*, 2006; Montzka and Fraser, 2003). MeBr concentrations have also decreased, but the matter is greatly complicated by the fact that only about 30% of the MeBr released is man made and therefore subject to direct control.

B. Biogeochemical Cycle of Monohalomethanes

The majority of methyl halides are produced naturally at the Earth's surface. Of major concern is the increased concentration and flux of atmospheric methyl halides (particularly MeBr) over the past 50–100 years, resulting from changes in the magnitude of sources and sinks. MeBr and MeCl react with atmospheric hydroxyl (OH) radicals and this oxidation represents their primary removal mechanism from the troposphere. The balance between release at the Earth's surface and loss in the troposphere results in sufficient atmospheric lifetimes (Table I) such that significant quantities of these compounds are able to reach the stratosphere where they can react with ozone. By contrast, MeI is removed more rapidly by photolysis in the troposphere and its shorter lifetime limits its impact on stratospheric ozone. In part this is due to the chemical reactivity of methyl halides, which increases with the atomic number of the halide ion (e.g., MeF < MeCl < MeBr < MeI). Uncertainty, however, clouds our understanding of the budgets of methyl halides. Currently, known sinks of MeBr and MeCl outweigh sources by about 35% (Montzka and Fraser, 2003). The budget for MeI is balanced, although there are large uncertainties in estimates of the oceanic source and the photolysis sink (Cox *et al.*, 2005).

C. Anthropogenic Sources

Methyl halides are manufactured for a variety of purposes by reacting acids, containing halogens derived from salt, with methanol from natural gas. Anthropogenic MeBr is used as a preplant and postharvest fumigant for crops in much of the world today (Table II). It is further used to fumigate structures for pest control and for quarantine fumigation of commodities shipped worldwide. In addition, MeBr can be used in treating buildings or vehicles contaminated with spores of *Bacillus anthracis* as a possible counterterrorism measure (R. Scheffrahn personal communication), although it was commonly used as far back as the 1950s to decontaminate animal hides prior to their handling by tanners. MeI is beginning to replace MeBr as a soil fumigant because it

TABLE II

ANTHROPOGENIC SOURCES OF ATMOSPHERIC METHYL HALIDES IN Gg year^{-1} (BEST ESTIMATES)

Source	CH_3Cl	CH_3Br	CH_3I
Biomass burning[a]	650	29	14
Fumigation—soils[b]	N.A.	26.5	Increasing
Fumigation—structural[b]	N.A.	2	N.A.
Fumigation—commodity[b]	N.A.	12.3	N.A.
Fossil fuel burning	105[c]	5[d]	20[e]
Industrial[f]	10		
Incineration[f]	45		

[a]Andreae and Merlet (2001).
[b]Kurylo *et al.* (1999).
[c]McCulloch *et al.* (1999); MeCl source is coal.
[d]Montzka and Fraser (2003); MeBr source is leaded gasoline.
[e]Orlando (2003).
[f]Keene *et al.* (1999).
N.A., not applicable.

is less hazardous to stratospheric ozone (Gan *et al.*, 1997). MeCl and MeBr are used industrially to manufacture other chemicals but little of this is released to the atmosphere. The largest strictly anthropogenic emissions of methyl halides derive from combustion of fossil fuels, particularly coal for MeCl and leaded gasoline for MeBr. These sources are generally comparable to or smaller than atmospheric inputs from biomass burning (Table II).

D. NATURAL CHEMICAL SOURCES AND SINKS

A fraction of methyl halides are created or destroyed naturally by chemical processes. This fraction may be large, as in the case of MeI production by photochemical reactions in surface seawater (Moore and Zafiriou, 1994; Richter and Wallace, 2004). Similarly, a large quantity of MeCl is produced in soils by reaction of plant-derived pectin with inorganic Cl^- (Hamilton *et al.*, 2003). A significant but unknown quantity of methyl halides is produced by the oxidation of soil organic matter in the presence of ferric iron and halides in soil and sediments (Keppler *et al.*, 2000). In aqueous environments, methyl halides are transformed by nucleophilic substitution of one halide for another, known as halide exchange (Jeffers and Wolfe, 1996; Lovelock, 1975;

Zafiriou, 1975). Hydrolysis, a similar chemical process involving nucleophilic substitution with water or hydroxide ion (OH^-), results in the removal of methyl halide and the production of methanol (Elliott and Rowland, 1995). The impact of these abiotic reactions remains unquantified, but they are of importance in the balance of methyl halides in soil (Bill *et al.*, 2002a,b) and seawater (Baesman and Miller, 2005).

E. Natural Biological Sources

By far, the largest sources of methyl halides to the atmosphere are the result of naturally occurring biological activity on the Earth's surface. This review examines the role that biology plays in affecting the balance between production and consumption of methyl halides in aquatic and terrestrial environments. Methyl halides are produced by a variety of cyanobacteria and marine algae, including phytoplankton (Scarratt and Moore, 1998) and macroalgae (Manley and Dastoor, 1987), as well as some terrestrial plants (Gan *et al.*, 1998a; Redeker *et al.*, 2000) and fungi (Harper, 1985; Saxena *et al.*, 1998). In algae, higher plants, and fungi, methylation of inorganic Br^- or Cl^- by *S*-adenosyl-L-methionine (SAM) to form *S*-adenosylhomocysteine and MeBr or MeCl is catalyzed by methyltransferase enzymes (Attieh *et al.*, 1995; Wuosmaa and Hager, 1990). Methyl halides are produced in large quantities by halophilic plants growing in salt marshes (Ni and Hager, 1999; Rhew *et al.*, 2000). However, this production is not particularly effective at removing excess halides. Rather, methyl halide production may simply be a metabolic coincidence resulting from the proximity of halide ions and methyltransferase enzymes within the plant cell (Manley, 2002). Production of methyl halides may provide a competitive advantage to some microorganisms, particularly fungi, through direct antimicrobial effects on their competitors/predators or by providing substrate for the biosynthesis of secondary metabolites which facilitate lignin degradation (Harper, 2000).

F. Natural Biological Sinks

Consumption of methyl halides is, likewise, largely under the control of biological processes occurring on the Earth's surface. In anaerobic sediments containing free sulfide, MeBr and MeCl may be transformed chemically to methanethiol (CH_3SH) or dimethylsulfide [$(CH_3)_2S$], which are substrates for both sulfate-reducing bacteria and methanogenic archaea (Oremland *et al.*, 1994). However, few anaerobic zones are in direct contact with the atmosphere at the Earth's surface, hence the largest

biological sinks for atmospheric methyl halides exist in aerobic environments. Lobert *et al.* (1995) demonstrated that the oceans were a net sink for atmospheric MeBr and subsequent work by Pilinis *et al.* (1996) suggested that biological activity in the oceans was responsible for some of the uptake. Shorter *et al.* (1995), working with a diversity of live soils, used flux measurements and additions of antibiotics to demonstrate that globally scaled MeBr consumption from the troposphere could be attributed to the metabolic activities of aerobic bacteria living within the tested soils. These results were significant in that they demonstrated a clear biological consumption of realistic tropospheric-mixing levels of MeBr, which are about 10–12 ppt. No other previous research on microbial trace gas consumption had dealt with microbial metabolism at such low concentrations. The ongoing assumption was that the affinity of oxidative enzymes for such rare trace gases would not be operative below some threshold boundary of say 100 ppb.

G. Stable Isotope Mass Balances and Fractionation

It was previously mentioned that large uncertainties exist in the magnitude of the terms comprising the global budgets of methyl halides. These uncertainties may be reduced by constructing stable carbon isotope mass balances. McCauley *et al.* (1999) first suggested constraining the atmospheric budgets of MeBr by analyzing the carbon isotopic composition of major sources and the fractionation associated with sinks (i.e., removal mechanisms). They determined the $\delta^{13}C$ value for industrial MeBr (–54.4‰ vs Vienna Peedee Belemnite; VPDB). This group and others determined the isotopic composition of MeBr entering the atmosphere from salt marshes and soil fumigations (–43‰ and –48.7‰, respectively; Bill *et al.*, 2002a,b). They further measured the isotopic composition of MeBr in the atmosphere (–44.4‰ to –41.2‰; Bill *et al.*, 2004), which allows closer examination of the effect of sinks on the stable isotope mass balance of methyl halides (see below).

Similar progress has been made with global MeCl mass balances (Keppler *et al.*, 2005). MeCl derived from biomass burning is released to the atmosphere with a $\delta^{13}C$ value about 25‰ depleted from the starting plant material (Thompson *et al.*, 2002) or about –47‰ ± 12‰ versus VPDB. Tropical vegetation is a large source of MeCl (Yokouchi *et al.*, 2002) with low $\delta^{13}C$ value (–72.7‰ to –69.3‰; Harper *et al.*, 2003). Isotopically ^{13}C-depleted MeCl is also emitted by salt marsh plants (–62‰; Bill *et al.*, 2002a) and wood-rotting fungi (–43‰; Harper *et al.*, 2001). Keppler *et al.* (2004) reported that MeCl released by low-temperature heating of plant leaves was highly depleted in ^{13}C with values ranging from

−147‰ to −119‰. The source of MeCl was the reaction of plant pectin (a ^{13}C-depleted fraction of the methoxyl pool) with chloride ion. The stable C isotopic composition of MeCl in the atmosphere ranges from −44‰ to −30‰ (Tsunogai *et al.*, 1999) with a global average of −36.2 ± 0.3‰ (Thompson *et al.*, 2002).

Large C isotopic fractionations (up to 72‰ for MeBr and up to 50‰ for MeCl) have been observed for bacterial reactions that oxidize methyl halides (Kalin *et al.*, 2001; Miller *et al.*, 2001). Large discrimination in favor of ^{12}C over ^{13}C was observed during oxidation of methyl halides by pure cultures of *Aminobacter ciceronei* IMB-1 and *Leisingera methylohalidivorans* MB2. However, for unknown biochemical reasons, MeBr degradation by *Aminobacter lissarensis* CC495 proceeded without such C fractionation. Patterns of stable C isotope measurements over time were part of a suite of tools used to distinguish biological from chemical removal processes in soil microcosms (Miller *et al.*, 2004). In water, chemical removal by hydrolysis or halide exchange reactions results in large fractionation of carbon isotopes (33–57‰; Baesman and Miller, 2005), resulting in enrichment of ^{13}C in the remaining methyl halide. These sinks may be significant in soil and fresh water at the Earth's surface and especially in the ocean, however, the impact these sinks have on the isotopic composition of methyl halides released to the atmosphere requires further investigation.

The largest sink for atmospheric MeBr and MeCl is the reaction with OH in the troposphere, a process which removes much of the surface flux of these compounds. Studied in the laboratory, these reactions produce large isotope fractionations (^{13}C depletion) by as much as 59 ± 8‰ for MeCl (Gola *et al.*, 2005) and 66 ± 30‰ for MeBr (C. J. Nielsen personal communication). These observations reveal a conundrum. If the tropospheric sink terms are large, and these reactions significantly fractionate the remaining unreacted methyl halide pool by enriching it in ^{13}C, then the global average isotope values for atmospheric methyl halides should not be similar to the weighted means of all sources. Put as a question, why is there little effect of the combined sink terms on the isotopic composition of the unreacted methyl halides in the troposphere? Could it mean that there are significant unidentified sources of isotopically ''light'' methyl halides to the atmosphere? Or perhaps the large fractionation attributed to OH radical oxidation is valid for laboratory settings only, and does not apply to the chemically more complex troposphere? These questions must be addressed before isotope mass balances can be successfully used to constrain global budgets, but it nonetheless illustrates the utility of stable C isotope balances in shaping testable hypotheses.

II. Methyl Halide-Degrading Organisms

A. Bacterial Degradation of Methyl Halides by Methanotrophs and Nitrifiers

A wide variety of microorganisms capable of degrading methyl halides have been described. First reports of bacterial methyl halide degradation were based on observations that certain bacteria were able to co-oxidize, but not grow on methyl halides. Methanotrophs such as *Methylococcus capsulatus* (Bath), *Methylosinus trichosporium*, and *Methylomonas methanica* were able to co-oxidize MeCl using the enzyme methane monooxygenase even though they could not grow on methyl halides as a carbon source (Stirling and Dalton, 1979; Stirling *et al.*, 1979). It has also been shown that metabolism of MeCl enhanced growth of *Methylomicrobium album* BG8 on methanol. Conversion of MeCl to carbon dioxide occurred, suggesting that co-oxidation of methyl halides by particulate methane monooxygenase may provide metabolic intermediates that can be assimilated by methanotrophs (Han and Semrau, 2000). Ammonia-oxidizing bacteria were also shown to degrade methyl halides. Using a bromide selective electrode, Hyman and Wood demonstrated that washed cell suspensions of *Nitrosomonas europaea* were able to generate bromide ions from MeBr at rates that were much higher than rates of chemical hydrolysis of MeBr. The generation of bromide slowed down to chemical hydrolysis rates when the medium became anoxic (Hyman and Wood, 1984). Rasche and colleagues showed by monitoring headspace gas concentrations that *N. europaea*, *Nitrosococcus oceani*, and *Nitrosolobus multiformis* degraded MeBr. Inhibition of MeBr degradation in these strains by allylthiourea, a specific inhibitor of ammonia monooxygenase (AMO), suggested that AMO was the enzyme responsible for MeCl degradation (Rasche *et al.*, 1990).

B. Diversity and Distribution of Bacteria Capable of Growth on Methyl Halides as a Carbon and Energy Source

MeCl and MeBr have both been used successfully in enrichment experiments resulting in the isolation of a variety of bacterial cultures that can grow on methyl halides (Table III). Methyl halide-degrading bacteria have been isolated from a range of environments, including agricultural, woodland and industrially contaminated soils, activated sludge, lake sediments, estuarine and seawater samples, suggesting that methyl halide-degrading bacteria are ubiquitous in the environment (McAnulla *et al.*, 2001a). Bacteria growing on methyl halides are mostly

TABLE III

PROPERTIES OF BACTERIAL ISOLATES CAPABLE OF GROWTH ON METHYL HALIDES

	Hyphomicrobium strain MC1	Strain MC	*Hyphomicrobium chloromethanicum* CM2	*Methylobacterium chloromethanicum* CM4	*Aminobacter ciceronei* IMB-1	*Aminobacter lissarensis* CC495	*Pseudomonas aeruginosa* NB1	*Leisingera methylohalidivorans* MB2	Strain LIS-3	*"Roseovarius"* strain 179	*"Ruegeria"* strain 198	*"Roseovarius"* strain 217
General properties												
Source	Sludge from industrial sewage plants	Activated sludge, sewage digester, Stuttgart, Germany	Soil at petrochemical factory, Tatarstan, Russia	Soil at petrochemical factory, Tatarstan, Russia	Fumigated strawberry farm soil in Irvine, California, USA	Beech woodland soil, County Down, Northern Ireland	Enrichment culture from activated sludge	Marine tide pool, coast of central California, USA	Long Island Sound, USA	Coastal seawater, Achmelvich Bay, Scotland	Coastal seawater, Achmelvich Bay, Scotland	English Channel seawater, off Plymouth, England
Gram staining	Gram negative	Gram positive	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Enrichment/ isolation reference	Hartmans *et al.*, 1986	Traunecker *et al.*, 1991	Doronina *et al.*, 1996	Doronina *et al.*, 1996	Connell Hancock *et al.*, 1998	Coulter *et al.*, 1999	Freedman *et al.*, 2004	Schaefer *et al.*, 2002	Hoeft *et al.*, 2000	Schäfer *et al.*, 2005	Schäfer *et al.*, 2005	Schäfer *et al.*, 2005
Description reference		–	McDonald *et al.*, 2001	McDonald *et al.*, 2001	McDonald *et al.*, 2005	McDonald *et al.*, 2005	–	Schaefer *et al.*, 2002		–	–	–
Culture collection no.	–/strain was lost	–	VKM B-2176T; NCIMB 13687T	VKM B-2223T; NCIMB 13688T	ATCC 202197; CIP 108660; CCUG 50580	NCIMB 13798; CIP 108661; CCUG 50579	–	ATCC BAA-92	–	–	–	–

Type	Facultative methyl-otroph	Homo-acetogen	Facultative methyl-otroph	Facultative methyl-otroph	Facultative methyl-otroph	Facultative methyl-otroph	Facultative methyl-otroph	Facultative methyl-otroph	Facultative methyl-otroph	Facultative methyl-otroph	Facultative methyl-otroph	Facultative methyl-otroph
Relation to oxygen	Aerobic	Strictly anaerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic, can denitrify	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic
Isolation substrate(s)	MeCl	MeCl	MeCl	MeCl	MeBr	MeCl	MeCl	MeBr	MeBr and trimethyl-amine	MeBr	MeBr	MeBr
Metabolism (growth substrates, methyl halide oxidation)												
MeCl	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	n.a.	Growth	Growth	Growth
MeBr	n.a.	n.a.	Growth	Oxidation	Growth	Growth	n.a.	Growth	Growth	Growth	Growth	Growth
MeI	n.a.	n.a.	Oxidation	Oxidation	Growth	Oxidation	n.a.	Growth	n.a.	n.a.	n.a.	n.a.
Other growth substrates	Methanol, formate, ethanol	Carbon monoxide, H_2/CO_2, methoxylated aromatic compounds	Methanol, formate, methyl-amine, ethanol	Methylamine, succinate, fumarate	Methylamine, range of sugars and organic acids	Methylamine, range of sugars and organic acids	Methanol, ethanol, acetate, glucose, Luria Bertani medium	Glycine betaine, methionine, yeast extract, marine broth (Difco, 2216)	Dimethyl-sulfide, di- and trimethyl-amine, glycine betaine, glucose, acetate	Glycine betaine, methionine, acetate, citrate, marine broth (Difco, 2216)	Glycine betaine, acetate, citrate, marine broth (Difco, 2216)	Methylamine, di- and trimethyl-amine, formate, glycine betaine, acetate, marine broth (Difco, 2216)
Presence of *cmuA*	n.a.	n.a.	Yes	Yes	Yes	Yes	n.a.	No	n.a.	Yes	Yes	No

n.a., data not available.

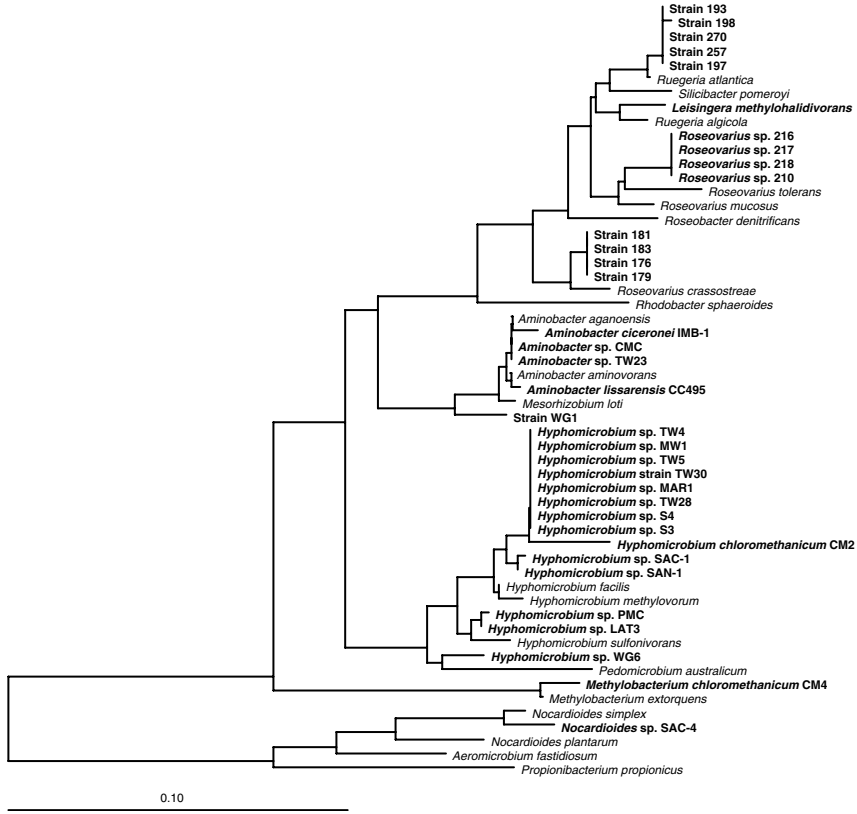

FIG. 1. Neighbor joining tree based on partial 16S rRNA gene sequences showing the diversity of bacteria capable of utilizing methyl halide as a growth substrate. Methyl halide-degrading bacteria are shown in bold type, the scale bar corresponds to 10% sequence divergence.

members of the *Alpha-Proteobacteria*, but also of the *Gamma-Proteobacteria* and the *Actinobacteria* (Fig. 1).

1. *Isolates from Activated Sludge*

Bacterial growth on MeCl as a sole carbon source was first reported for *Hyphomicrobium* strain MC1 (Hartmans *et al.*, 1986) which was isolated from activated sludge. Unfortunately this strain has been lost. Activated sludge was also the source of strain MC, a strictly anaerobic Gram-positive, homoacetogenic bacterium, which grew on MeCl as sole source of carbon and energy (Traunecker *et al.*, 1991).

2. *Soil and Freshwater Isolates*

Doronina *et al.* (1996) isolated *Hyphomicrobium* and *Methylobacterium* strains from contaminated soil at the site of a petrochemical factory in Russia. These strains assimilated formaldehyde using the serine cycle (Anthony, 1982) and were able to grow on MeCl as sole source of carbon and energy using an unknown inducible enzyme system for degradation of MeCl.

Subsequently further facultative methylotrophs capable of methyl halide degradation were isolated from the terrestrial environment. Strain IMB-1 was obtained from a southern Californian strawberry field soil that had been subject to fumigation with MeBr (Miller *et al.*, 1997), and was initially identified as being closely related to members of the genus *Rhizobium*. It was isolated by using MeBr as its sole source of carbon and energy (Miller *et al.*, 1997). The high toxicity of MeBr was circumvented by making a series of pulsed, low concentration additions of MeBr to the gas phase (typically ~0.5%) over the course of the incubations. A similar strategy was used to demonstrate growth of this isolate on MeI (Connell Hancock *et al.*, 1998). A related isolate that grew on MeCl, strain CC495 was obtained from a woodland soil in Northern Ireland by Coulter *et al.* (1999). These isolates have been characterized as novel species *A. ciceronei* IMB-1 and *A. lissarensis* CC495 (McDonald *et al.*, 2005). Additional strains of *Hyphomicrobium* were isolated from a variety of woodland soils and also from lake sediments (McAnulla *et al.*, 2001a). The only aerobic Gram-positive strain growing on MeCl that has been described was a *Nocardioides* strain (strain SAC-4), isolated from a woodland soil (McAnulla *et al.*, 2001a). Unfortunately this isolate has been lost. An isolate of *Pseudomonas aeruginosa*, strain NB1, was shown to degrade MeCl under aerobic and denitrifying conditions (Freedman *et al.*, 2004).

3. *Marine Methyl Halide Utilizers*

Despite the importance of the marine environment as a sink in the global cycle of methyl halides (Yvon-Lewis and Butler, 1997, 2002), only a few marine strains growing on methyl halides have been isolated. Although a *Hyphomicrobium* strain was isolated from the Severn estuary in the UK (McAnulla *et al.*, 2001a), *Hyphomicrobium* spp. are not typical marine bacteria and so the isolation of *L. methylohalidivorans* MB2 from a tidal pool off the Californian coast can be considered the first marine strain growing on MeBr (Goodwin *et al.*, 1998). Strain MB2 was characterized as a facultative methylotroph belonging to the *Roseobacter* clade (Schaefer *et al.*, 2002), an abundant group of microorganisms in

seawater (González and Moran, 1997). Subsequently a further 13 facultative methylotrophic strains growing on MeBr, affiliated with the *Roseobacter* clade, were isolated from the Scottish coast and the English Channel. These strains belonged to three different species of the *Roseobacter* clade and were most closely related to members of the genera *Roseovarius* and *Ruegeria* (Schäfer *et al.*, 2005).

Further MeBr-degrading strains were obtained from seawater samples but were not identified by 16S rRNA sequencing (Hoeft *et al.*, 2000). One of these isolates, strain LIS-3, grew poorly on MeBr, another isolate, strain FV co-oxidized MeBr and could not grow on MeBr (Hoeft *et al.*, 2000). Similarly, a strain related to the *Sphingomonadaceae*, strain Oxy6, cometabolized MeBr (Goodwin *et al.*, 2005). Strain Oxy6 was obtained from a toluene enrichment and MeBr consumption appeared to be cometabolic and inhibited by toluene.

III. Biochemistry and Genetics of Methyl Halide Degradation

A. Metabolism of Methyl Halides by Bacterial Isolates

The work of Hartmans *et al.* (1986) on the first isolate that grew on MeCl, *Hyphomicrobium* MC1, showed that MeCl degradation in strain MC1 released chloride ions in a stoichiometric manner. Methane was not oxidized by the strain, which excluded the possibility that methane monooxygenase was responsible for MeCl degradation. Resting cells of strain MC1 grown on MeCl dechlorinated MeCl with concomitant oxygen consumption, whereas methanol-grown cells did not. Methanol-grown cells had high rates of methanol oxidation, while MeCl-grown cells had low methanol oxidation rates. It was suggested that formaldehyde was an intermediate in MeCl metabolism and that a monooxygenase could be involved (Hartmans *et al.*, 1986). The mechanism of MeCl dehalogenation however was not identified in this strain and since the strain was lost, further biochemical analysis was not possible.

As with strain MC1, methyl halide metabolism generally appears to be inducible in methylotrophs growing on these compounds. This trait has been described for several strains, including *Methylobacterium chloromethanicum* CM4 (Doronina *et al.*, 1996; Vannelli *et al.*, 1998), *Hyphomicrobium chloromethanicum* CM2, and the marine isolates *Roseovarius* sp. 217, *Ruegeria* sp. 198, and strain 179 (Schäfer *et al.*, 2005). Although cells of *A. ciceronei* IMB-1 grown in the absence of MeBr had constitutive MeBr degradation activity at low concentrations of MeBr (<20 nM), induction by MeBr was required for consumption of higher MeBr concentrations (100 μM and above) (Schaefer and

Oremland, 1999). MeBr-oxidation experiments with *L. methylohalidivorans* MB2 in the presence of the protein synthesis inhibitor chloramphenicol indicated rapid turnover of MeBr-degrading enzymes, even when exposed to tropospheric levels (12 ppt) of MeBr (Goodwin *et al.*, 2001). However, although for *A. ciceronei* IMB-1, the identity of the enzyme system responsible for MeBr consumption at tropospheric-mixing ratios has not been established, it is possible that the enzyme system could be identical to the enzymes responsible for consumption of higher MeBr concentrations. It is interesting to note that the kinetic parameters of MeBr consumption estimated at mixing ratios near tropospheric levels were similar to those derived from experiments using higher mixing ratios and batch incubations. Although K_s values were 10^2–10^3 times higher than tropospheric-mixing ratios, *A. ciceronei* IMB-1 and *L. methylohalidivorans* MB2 both could consume tropospheric levels of MeBr (Goodwin *et al.*, 2001).

B. The CmuA Pathway of Methyl Halide Degradation in *M. chloromethanicum* Strain CM4

A combination of elegant genetic and biochemical experiments led to the identification of the pathway of MeCl metabolism in *M. chloromethanicum* CM4 (Studer *et al.*, 1999, 2001, 2002; Vannelli *et al.*, 1998, 1999). Leisinger and coworkers introduced random mutations in *M. chloromethanicum* CM4 by transposon mutagenesis and screened for mutants which could not grow on MeCl, but which could still grow on methanol (Vannelli *et al.*, 1998). Analysis of the site of transposon insertions that lead to a loss of MeCl degradation revealed a number of genes that were (potentially) essential for growth on MeCl as a carbon source (Vannelli *et al.*, 1999). These included genes designated *cmuA*, *cmuB*, and *cmuC* that were predicted to encode methyltransferases, based on similarity shared with methyltransferases from methanogenic archaea. The gene *cmuA* encoded an unusual protein of 617 amino acids, which appeared to have two functionally distinct domains. While the N-terminal domain of CmuA had similarity to the coenzyme M methyltransferases (MtbA) involved in methanogenesis from methylamine in *Methanosarcina barkeri*, the C-terminal domain was similar to the monomethylamine corrinoid protein (MtmC) of the same organism, which accepts the methyl group from methylamine during growth on this compound (Vannelli *et al.*, 1999). Both CmuA and CmuB were purified from *M. chloromethanicum* strain CM4 and it was shown *in vitro* that CmuA and CmuB in combination were able to transfer the methyl group of MeCl to tetrahydrofolate (H_4F) (Studer *et al.*, 1999, 2001).

It was also shown that tetrahydromethanopterin did not accept the methyl group from CmuB *in vitro*. Other genes in the vicinity of *cmuA* and *cmuB* were essential for growth of *M. chloromethanicum* CM4 on MeCl. These additional genes, *metF* (encoding methylene-H_4F reductase), *folD* (encoding methylene-H_4F dehydrogenase/methenyl-H_4F cyclohydrolase), and *purU* (encoding formyl-H_4F hydrolase) were specifically induced during growth in the presence of MeCl and were transcribed from consensus promoters that differed from previously identified *Methylobacterium* promoters (Studer *et al.*, 2002).

On the basis of these genetic and biochemical data, a new C_1 utilization pathway was postulated for growth of CM4 on MeCl (Studer *et al.*, 2002) (Fig. 2), which in part is similar to the H_4F-linked pathway of formaldehyde oxidation. In this pathway, MeCl is dehalogenated by CmuA in the initial step and the methyl group is transferred to a corrinoid on the same polypeptide chain. CmuB, the secondary methyltransferase, then transfers the methyl group to H_4F yielding methyl-H_4F, which is further oxidized by 5,10-methylene-H_4F reductase to 5,10-methylene H_4F. This latter intermediate provides the formaldehyde for carbon assimilation by the serine cycle which is critical for the synthesis

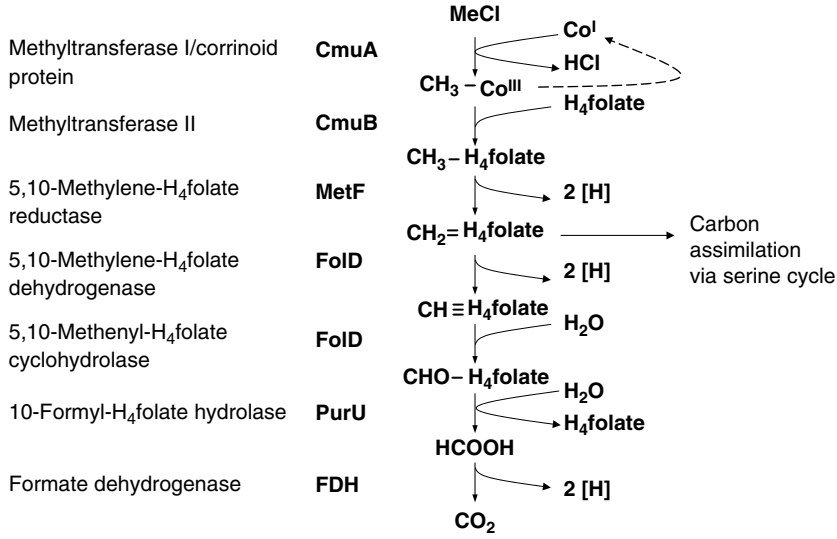

FIG. 2. The MeCl utilization pathway of *Methylobacterium chloromethanicum* CM4 as proposed by Vannelli and coworkers (modified from Vannelli *et al.*, 1999). Names of enzymes catalyzing steps in the degradation pathway are shown on the left of the diagram.

of multicarbon compounds from C_1 substrates (Anthony, 1982). However, 5,10-methylene H_4F can also be further oxidized to yield reducing equivalents for energy generation. This is accomplished by the bifunctional enzyme 5,10-methylene H_4F dehydrogenase/5,10-methenyl-H_4F cyclohydrolase (FolD) yielding 10-formyl-H_4F. In the final steps of the pathway, 10-formyl-H_4F is oxidized to formate by 10-formyl-H_4F hydrolase and formate is oxidized to carbon dioxide by formate dehydrogenase (Studer *et al.*, 2002). It is interesting to note that some of the potential energy gain from the oxidation of 10-formyl-H_4F is ''wasted'' by the hydrolase reaction, since the oxidation of this compound could also be coupled to generation of ATP by the enzyme formate-H_4F ligase (formyl-H_4F synthetase), if present in this organism.

1. *MeCl Metabolism in* A. lissarensis *CC495*

A 67-kDa halomethane:bisulfide/halide ion methyltransferase that was induced in *A. lissarensis* CC495 during growth on MeCl was purified by Coulter *et al.* (1999). The polypeptide transferred methyl groups from MeCl, MeBr, and MeI to a corrinoid bound on the same polypeptide chain. The polypeptide also exhibited transhalogenating activity by transferring the methyl group from methyl halides to a number of acceptor ions including I^-, HS^-, Cl^-, Br^-, NO_2^-, CN^-, and SCN^-. It was suggested that HS^- could be the physiological acceptor ion for methyl groups from methyl halides, since activities of methanethiol oxidase and formaldehyde dehydrogenase were detected in cell extracts of strain CC495 grown on MeCl, providing a route of C_1 metabolism to formate (Coulter *et al.*, 1999). Gene cloning and sequencing has identified this halomethane:bisulfide/halide ion methyltransferase as a homologue of CmuA (Warner *et al.*, 2005) (see below). Further genes implicated in methyl halide metabolism in *M. chloromethanicum*, *H. chloromethanicum*, and *A. ciceronei* were found upstream and downstream of the gene encoding CmuA in strain CC495 (Warner *et al.*, 2005). These findings suggest that methyl halide metabolism in strain CC495 might also proceed via H_4F, or alternatively, that different pathways for methyl halide degradation may exist even in the same organism which might operate under different physiological conditions (Warner *et al.*, 2005). This needs to be addressed in more detail in future studies.

C. Cloning and Sequencing of *cmu* Gene Clusters

To date, *cmu* gene clusters have been cloned and sequenced from six methyl halide-degrading strains, including *M. chloromethanicum* CM4, *H. chloromethanicum* CM2, *A. ciceronei* IMB-1, *A. lissarensis*

CC495, ''*Roseovarius*'' strain 179, and ''*Ruegeria*'' strain 198 (Borodina *et al.*, 2004; McAnulla *et al.*, 2001b; Schäfer *et al.*, 2005; Vannelli *et al.*, 1999; Warner *et al.*, 2005; Woodall *et al.*, 2001). A high degree of conservation of the structure of *cmu* clusters between methyl halide-degrading bacteria has been observed (Fig. 3). In most cases, the order of genes found in *cmu* clusters is conserved to some degree, the exception being *M. chloromethanicum* CM4 where *cmuB* and *cmuC* are on one transcriptional unit and *cmuA* is on a separate transcriptional unit (Studer *et al.*, 2002) (Fig. 3). A different organization of *cmu* genes was found in *H. chloromethanicum* CM2, *A. ciceronei* IMB-1, *A. lissarensis* CC495, and strains 179 and 198. Apart from *H. chloromethanicum* CM2, in which *folD* was found upstream of a *cmu* cluster, the gene clusters did not contain *cob* genes of cobalamin biosynthesis, *folC* and *folD*, nor *purU* encoding formyl H_4F hydrolase. However, this does not exclude the possibility that these may be located upstream or downstream of the partially sequenced gene clusters from these isolates. Sequencing of the *cmu* gene clusters from *H. chloromethanicum* CM2, *A. ciceronei* IMB-1, *A. lissarensis* CC495, strains 179, and 198 identified additional genes, *fmdB*, *paaE*, and *hutI* and the order of these genes was conserved. These genes were hypothesized to encode a transcriptional regulator (*fmdB*), a reductase (*paaE*), and an imidazolone propionase (*hutI*) (McAnulla *et al.*, 2001b). Although the role of these genes and their encoded polypeptides is unclear, it is possible that they have a role in the metabolism of methyl halides. Of the three additional open reading frames upstream of *cmuB* that were identified in *H. chloromethanicum* CM2 (note: these genes are not shown in Fig. 3), one was similar to a porin precursor from *Neisseria denitrificans*. The other two, however, did not have matches in the database.

As in *M. chloromethanicum* CM4, a gene (*metF*) encoding methylene-H_4F reductase was found downstream (3′) of *hutI* in *A. ciceronei* IMB-1, *H. chloromethanicum* CM2, and the marine strain ''*Roseovarius*'' sp. 179. In the marine strain ''*Ruegeria*'' sp. 198, *metF* was not found and sequence downstream (3′) of *hutI* contained ribonucleoside diphosphate reductase genes *nrdF* and *nrdA* orientated in the opposite direction to the open reading frames of the *cmu* gene cluster. The location of *metF* is therefore not conserved between these strains. In four of these gene clusters, *cmuC* was found upstream of *cmuA*, and additionally *cmuB* was found upstream of *cmuC* in *A. lissarensis* CC495 and *H. chloromethanicum* CM2. The high similarity between these gene

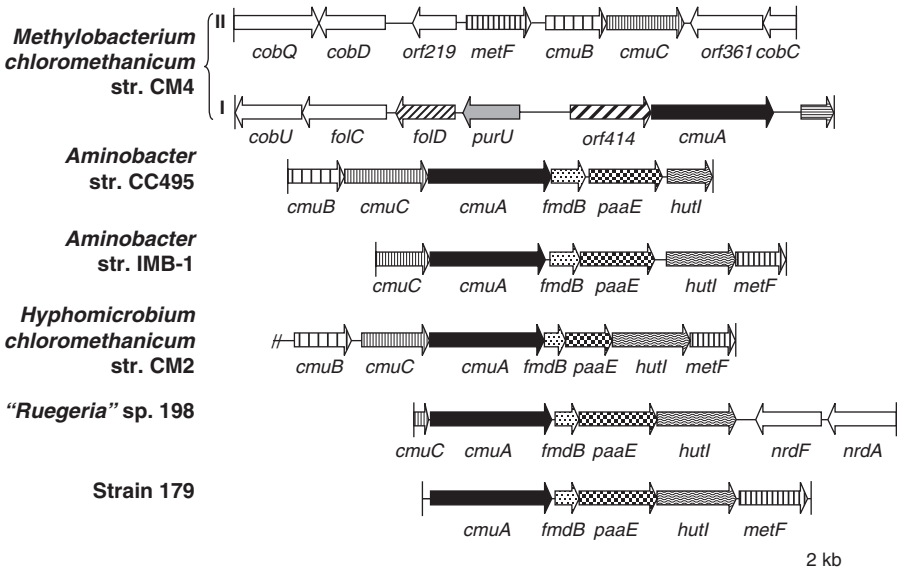

FIG. 3. Gene clusters of methyl halide-utilizing strains containing genes with proven and putative function in the CmuA methyl halide utilization pathway. Homologous genes have identical shading.

clusters suggests that the gene order *cmuBCA* may be conserved between all these gene clusters with the exception of *M. chloromethanicum* CM4. Further, cloning and sequencing work is required to investigate this in detail.

D. Mutational and Transcriptional Analysis of *cmu* Genes of *H. chloromethanicum* CM2

Initial cloning and sequencing of the *cmu* gene cluster of *H. chloromethanicum* CM2 was accomplished by McAnulla *et al.* (2001b). Further progress was made by Borodina *et al.* (2004) who successfully developed and applied a genetic system for strain *H. chloromethanicum* CM2, allowing delivery of transposon mutagenesis vectors by electroporation. Borodina and colleagues obtained a number of mini*Tn*5 insertion mutants that could not grow on MeCl. In one of these mutants, the transposon was inserted at the 3′ end of a gene encoding the putative imidazolone propionase HutI and 5′ of the start of the gene encoding methyl H_4F reductase (MetF) which overlapped with the end of *hutI*. A further eight transposon insertion mutants disrupted the gene encoding the putative methyltransferase CmuC. Although insertional inactivation of *cmuC* also produced a MeCl-minus mutant in *M. chloromethanicum* CM4, in *H. chloromethanicum* CM2, the mutation affected expression of CmuA as shown by SDS-PAGE analysis of cell extracts of the mutants. This was different to the findings in *M. chloromethanicum* CM4, where CmuA and CmuB were both expressed and functional in *cmuC*$^-$ mutants. However, mutation of *cmuC* led to a MeCl-minus phenotype in *M. chloromethanicum* CM4, suggesting that the CmuC methyltransferase has a role in methyl halide metabolism which has yet to be determined. In *H. chloromethanicum* CM2, the mutation in the *hutI-metF* region had a similar effect on expression of CmuA, again revealed by SDS-PAGE analysis, as did the mutation of *cmuB* (accomplished by marker exchange mutagenesis). This led to the suggestion that mutations in *cmuB*, *cmuC*, and *hutI-metF* exerted polar effects that abolished the expression of CmuA. Reverse transcriptase PCR experiments showed that the *cmuBCA-metF* operon was cotranscribed in *H. chloromethanicum* CM2 and tightly coregulated in a strictly MeCl-dependent manner, whereby the presence of MeCl led to the transcription of the cluster, even when methanol was also present during growth (Borodina *et al.*, 2004). The promoter of the operon has subsequently been identified by RACE PCR (rapid amplification of cDNA ends) and lies upstream of *cmuB* (Borodina, McDonald, and Murrell, unpublished data).

E. Evidence for Operation of the CmuA Pathway in MeCl- and MeBr-Degrading Bacterial Isolates

There is ample evidence that the isolates harboring *cmu* gene clusters degrade methyl halide using a pathway similar to that identified in *M. chloromethanicum* CM4. Mutational analysis of *cmu* cluster genes (i.e., *cmuB*, *cmuC*, *cmuB*, *metF*, and *purU*) in CM4 and *H. chloromethanicum* CM2 has shown that these genes are required for growth on methyl halides (Borodina *et al.*, 2004; Studer *et al.*, 2002; Vannelli *et al.*, 1999). The enzyme activities of this pathway have been demonstrated in CM4 and CM2 (McAnulla *et al.*, 2001b; Vannelli *et al.*, 1999) and CmuA has been purified from *M. chloromethanicum* CM4 (Studer *et al.*, 2001) and *A. lissarensis* CC495 (Coulter *et al.*, 1999). Expression of CmuA during growth on methyl halides has been demonstrated by SDS-PAGE in the soil isolates *M. chloromethanicum* CM4, *H. chloromethanicum* CM2, and *A. lissarensis* CC495 (Coulter *et al.*, 1999; McAnulla *et al.*, 2001b; Studer *et al.*, 2001; Vannelli *et al.*, 1998). CmuA was identified as a 67-kDa polypeptide that was expressed during growth on MeBr in the marine isolates ''*Roseovarius*'' strain 179 and ''*Ruegeria*'' strain 198. This has been confirmed by mass spectrometry (Schäfer *et al.*, 2005).

Identical polypeptide profiles of biomass grown on MeCl and MeBr of ''*Ruegeria*'' strain 198 confirmed that the same pathway is responsible for degradation of both MeBr and MeCl. In *H. chloromethanicum* CM2, transcription of the *cmu* gene cluster was controlled by MeBr in a similar fashion as with MeCl (Borodina, McDonald, and Murrell, unpublished data). Although no detailed investigation of MeI degradation has been reported for any of the methyl halide-degrading isolates, it seems reasonable to assume that the CmuA pathway could also be responsible for degradation of MeI, especially since it has been shown that CmuA from *A. lissarensis* CC495 was able to transfer the methyl group from MeCl, MeBr, and MeI to a variety of acceptor ions (Coulter *et al.*, 1999).

It is likely that environmental concentrations of methyl halides are sufficient to induce the enzyme system, although it might be difficult to observe the 67-kDa band of CmuA by SDS-PAGE when cells are grown at atmospheric methyl halide-mixing ratios, since the amount of this peptide may be low under such growth conditions. Whether natural picomolar concentrations of methyl halides are indeed sufficient to induce the CmuA pathway could be determined by comparison of wild-type and mutant strains of methyl halide utilizers. At present, mutants are only available for *M. chloromethanicum* CM4 and

H. chloromethanicum CM2. Mutational analysis has not yet been accomplished for the *Aminobacter* strains.

It is important to note that growth of methyl halide-degrading bacteria on more favorable carbon sources did not suppress methyl halide oxidation. *H. chloromethanicum* CM2 could grow on methanol and MeCl simultaneously and expression of CmuA was shown by SDS-PAGE (Borodina *et al.*, 2004). Similarly, the expression of CmuA in the marine strain 179 growing on betaine and MeBr was demonstrated by mass spectrometry (Schäfer *et al.*, 2005).

F. Alternative Methyl Halide Degradation Pathways

There is some evidence to suggest that the CmuA pathway is not the only methyl halide utilization pathway (see also Table IV). Extensive Southern blotting and gene probing of *L. methylohalidivorans* MB2 with *cmuA* probes, prepared from terrestrial isolates and from a closely related marine isolate, ''*Roseovarius*'' 179, failed to show any indications of a homologue of *cmuA* in MB2 (Schäfer *et al.*, 2005; Warner, 2003); it is therefore possible that *L. methylohalidivorans* MB2 has a different pathway for methyl halide degradation. Another *Roseobacter* clade isolate, ''*Roseovarius*'' 217 strain did not induce a 67-kDa polypeptide during growth on methyl halides. As with *L. methylohalidivorans* MB2, Southern blotting and gene probing failed to highlight a *cmuA* homologue in strain 217 (Schäfer *et al.*, 2005). Strain LIS-3 isolated by Hoeft *et al.* (2000) appeared to degrade MeBr by a pathway involving methyltransferase reactions, this was concluded from the inhibition of MeBr oxidation by chloroform (Hoeft *et al.*, 2000). Coulter *et al.* (1999) reported that the homologue of the CmuA methyltransferase in *A. lissarensis* CC495 was not inhibited by chloroform. It would therefore seem possible that strain LIS-3 used an enzyme system to degrade MeBr which is different to that in *A. lissarensis*.

IV. Microbial Ecology of Methyl Halide-Degrading Bacteria

A. Development of Functional Gene Markers for CmuA Pathway Methylotrophs

PCR primers for the amplification of *cmuA* were first developed by McAnulla *et al.* (2001b) based on *cmuA* sequences from *M. chloromethanicum* CM4 and *H. chloromethanicum* CM2. The gene *cmuA* was chosen as a key functional molecular marker for targeting methyl halide-utilizing bacteria since it encodes the enzyme carrying out the

TABLE IV

SUMMARY OF OBSERVATIONS SUGGESTING DIFFERENT PATHWAYS OF METHYL HALIDE DEGRADATION

Pathway/key enzyme	Representative isolates	Inhibitors/evidence	References
Methane oxidation/ methane monooxygenase (co-oxidation)	*Methylococcus capsulatus* (Bath)	Acetylene	Stirling and Dalton, 1979
	Methylosinus trichosporium		Stirling *et al.*, 1979
	Methylomonas methanica		Han and Semrau, 2000
	Methylomicrobium album		
Ammonia oxidation/ ammonium monooxygenase (co-oxidation)	*Nitrosomonas europaea*	Allylthiourea	Rasche *et al.*, 1990
	Nitrosococcus oceani		
	Nitrosolobus multiformis		
cmu methyltransferase pathway/CmuA	*Methylobacterium chloromethanicum* CM4	No inhibition by chloroform, methyl *tert* butyl ether, or propyl iodide	Coulter *et al.*, 1999
	Hyphomicrobium chloromethanicum CM2		Doronina *et al.*, 1996
	Aminobacter ciceronei IMB-1		Connell Hancock *et al.*, 1998
	Aminobacter lissarensis CC495		Schäfer *et al.*, 2005
	''*Roseovarius*'' sp. 179		
	''*Ruegeria*'' sp. 198		
Methyltransferase	Strain LIS-3	Chloroform	Hoeft *et al.*, 2000
Monooxygenase (co-oxidation)	Strain FV	Methyl *tert* butyl ether	Hoeft *et al.*, 2000
Uncharacterized aromatic hydrocarbon degradation pathway (co-oxidation)	*Sphingomonas* sp. Oxy6	Toluene	Goodwin *et al.*, 2005
Uncharacterized pathway	''*Roseovarius*'' sp. 217	No inhibition by chloroform, methyl *tert* butyl ether, or propyl iodide	Schäfer *et al.*, 2005
		CmuA not detected by SDS-PAGE, *cmuA* gene absent (Southern hybridization, genome sequence)	Schäfer and Murrell, unpublished

first defining step in the methyl halide utilization pathway. The unique domain structure of CmuA was exploited by generating PCR primer sequences that would anneal to target sites in regions coding the N-terminal methyltransferase domain and the C-terminal corrinoid-binding domain. This was predicted to increase the specificity of the PCR primer set. While specific PCR products were obtained with six methyl halide-degrading *Hyphomicrobium* isolates, the primers failed to amplify a product from *A. ciceronei* IMB-1. From Southern blotting and gene probing this strain was known to possess *cmuA* but its sequence was not yet known. New PCR primers were designed, again placing forward primers in the methyltransferase domain and reverse primers in the part of *cmuA* encoding the corrinoid-binding domain. Sequencing of PCR products confirmed that one of these primer sets amplified *cmuA* fragments from all *Hyphomicrobium* strains and *A. ciceronei* IMB-1. No amplification was observed with non-methyl halide-degrading isolates. This primer pair was used to amplify *cmuA* genes from an MeCl-degrading soil enrichment culture. Cloning and sequencing revealed that three sequence types were obtained, one being closely related to *H. chloromethanicum* CM2, another identical to the sequence from *A. ciceronei* IMB-1, and a third sequence that was only 91% identical to CmuA from *H. chloromethanicum* CM2 (McAnulla *et al.*, 2001b).

PCR primers for *cmuA* were further refined with the availability of the *cmu* gene clusters from *A. ciceronei* IMB-1 and *A. lissarensis* CC495 which were also cloned and sequenced (Warner, 2003, 2005; Woodall *et al.*, 2001) and have since proven useful in the analysis of the diversity of this gene in various enrichment cultures, bacterial isolates, and environmental samples (Borodina *et al.*, 2005; Miller *et al.*, 2004; Schäfer *et al.*, 2005). The phylogenetic tree in Fig. 4 illustrates the diversity of the gene encoding CmuA from various bacterial isolates and sequences obtained after direct amplification of *cmuA* from a variety of environmental samples.

B. Stable Isotope Probing of Methyl Halide Degradation in Soils

Stable isotope probing (SIP) is an experimental method that links the degradation of ^{13}C-labeled growth substrates to the phylogenetic diversity of bacteria that use these substrates *in situ* (Radajewski *et al.*, 2000). For an overview of the application of SIP, the reader is referred to reviews (Dumont and Murrell, 2005; Evershed *et al.*, 2006; Friedrich, 2006; Radajewski *et al.*, 2003; Whiteley *et al.*, 2006). Previously, Boschker *et al.* (1998) used stable isotopes to label phospholipid fatty

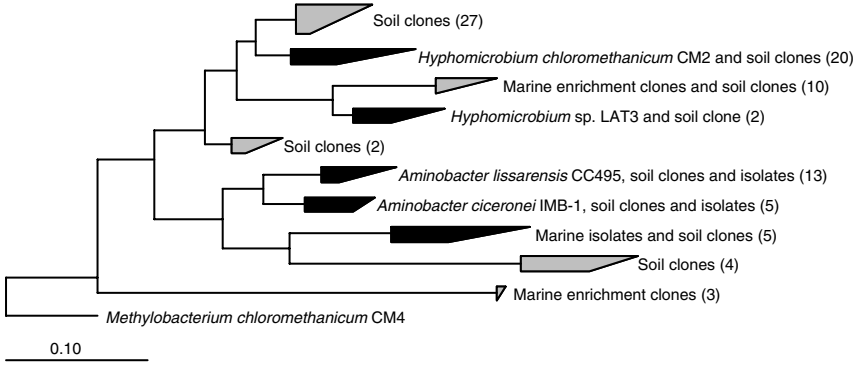

FIG. 4. Phylogenetic tree based on neighbor joining analysis of amino acid sequence alignment of CmuA sequences (methyltransferase I/corrinoid-binding protein) from methyl halide-degrading bacteria and environmental samples. Closely related sequences have been grouped, whereby those groups without cultivated representatives have been shaded grey. Numbers in parentheses indicate the number of sequences of each group. Labels left of groups indicate representative sequences, including cultivated members. The scale bar represents 10% sequence divergence.

acids as biomarkers of microorganisms in anoxic sediments. SIP targeting nucleic acids was first developed as DNA-SIP (stable isotope probing based on analysis of DNA) with methanol-utilizing methylotrophs (Radajewski *et al.*, 2000). Since then, the application of SIP has been further developed using both DNA and RNA and a variety of ^{13}C-labeled substrates. The principle of SIP is based on the assimilation of the ''heavy'' carbon into the biomass of the active population in the environment, which is supplied as >99% ^{13}C-labeled substrate in microcosm experiments. The biomass of ^{13}C-substrate assimilating organisms becomes ^{13}C-labeled, including the nucleic acids; organisms that do not utilize the ''heavy'' substrate remain unlabeled. The nucleic acids of labeled and unlabeled organisms can be physically separated by density gradient centrifugation and ''light'' and ''heavy'' DNA or RNA fractions can then be studied using a variety of molecular microbial ecology methods.

1. *Stable Isotope Probing of Agricultural Soils*

DNA-SIP was used successfully with MeCl and MeBr in agricultural soils (Miller *et al.*, 2004) to identify active populations of methyl halide-utilizing bacteria. Light and heavy density gradient fractions of DNA extracted from soil microcosms that had been exposed to ^{13}C-MeCl or ^{13}C-MeBr were analyzed for bacterial diversity using denaturing gradient gel electrophoresis (DGGE) of partial bacterial

16S rRNA genes and by sequencing of cloned 16S rRNA genes. The functional diversity of methyl halide-utilizing organisms was analyzed by clone library analysis of *cmuA* genes. DGGE analysis of heavy DNA fractions revealed different community fingerprints for microcosms that had been exposed to MeCl and MeBr, indicating that the populations degrading these substrates *in situ* may be different. Although this might not necessarily be expected, based on the observation that the CmuA pathway is capable of MeBr and MeCl degradation, the fact that not all methyl halide-degrading bacteria are equally capable of growth on MeCl and MeBr (and MeI) (see Table III) suggests that bacteria may have differing capacity to deal with these substrates of differing toxicity. Analysis of 16S rRNA gene clone libraries of the heavy DNA fraction failed to show sequences matching those of known methyl halide-utilizing isolates, such as *Methylobacterium*, *Hyphomicrobium*, and *Aminobacter*. The dominant sequence types that were recovered from the heavy DNA fractions of MeBr soil microcosms were related to *Burkholderia*, while the dominant clone sequences in heavy DNA fractions of MeCl soil microcosms were related to *Rhodobacter*, *Lysobacter*, and *Nocardioides*. Further phylotypes were detected in both MeCl and MeBr microcosms; however, the diversity detected by the clone libraries was greater for MeCl soil microcosms compared to MeBr microcosms, possibly due to the higher toxicity of MeBr. The detection of 16S rRNA gene sequences similar to *Nocardioides* was particularly interesting since the sequence was similar to a methyl halide-degrading isolate previously isolated from woodland soil (McAnulla *et al.*, 2001a) which was lost, confirming that these bacteria may play a role in cycling of methyl halides. The results of the study by Miller *et al.* (2004) demonstrated that the dominant types of methyl halide-utilizing bacteria in agricultural soils may be different from the extant isolates, and suggested that the phylogenetic diversity of methyl halide-degrading bacteria is not represented by the strains currently in culture. The diversity of *cmuA* genes in the heavy DNA fraction was also analyzed. The *cmuA* sequences obtained from the SIP experiment belonged to four distinct clades, which were similar to *cmuA* from *A. lissarensis* CC495, a second group of sequences more distantly related to *cmuA* sequences from *Aminobacter* species, a deeply branching group of sequences, and one clone which was similar to *cmuA* sequences of *Hyphomicrobium* species. The observation that the overall diversity of 16S rRNA libraries was higher than that of the *cmuA* clone libraries might be related to a certain bias in the primer sequences that were designed based on the known *cmuA* genes from isolates that only represented *Alpha-Proteobacteria.* They might therefore fail to

amplify more divergent *cmuA* genes (Miller *et al.*, 2004). Furthermore, it is possible that some of the methyl halide-utilizing populations, detected in 16S rRNA gene libraries, could have a different pathway of methyl halide degradation.

2. *Stable Isotope Probing of Woodland and Forest Soils*

In a similar study, Borodina and colleagues used DNA-SIP to analyze the diversity of MeCl-utilizing populations in a variety of soils. The SIP approach was complemented by enrichment and isolation of MeCl-degrading bacteria. Isolates were identified by sequencing of their 16S rRNA genes and included strains closely related to *Aminobacter* and *Mesorhizobium*. The majority of the isolates however were identified as *Hyphomicrobium* species. The diversity of *cmuA* was also analyzed using DNA directly extracted from soil samples, DNA extracted from enrichment cultures and ^{13}C-labeled DNA from SIP experiments. A major group of *cmuA* sequences belonged to a clade that did not have cultivated representatives, and included cloned *cmuA* sequences obtained from DNA of woodland, forest, and garden soil, as well as *cmuA* clones from enrichments inoculated with garden soil. Sequences of *cmuA* closely related to *cmuA* of *Aminobacter* were identified in DNA from garden soil enrichments and DNA extracted from wood soil. Another clade was composed of *Hyphomicrobium* species *cmuA* sequences and related sequences which were detected in garden soil DNA and also as a dominant *cmuA* sequence type in clone libraries of the heavy fraction of a wood soil DNA-SIP experiment. Surprisingly, sequences of other dominant cloned *cmuA* genes from the SIP heavy fraction were similar to those of marine *cmuA* sequences obtained from MeBr enrichment cultures of coastal seawater from the English Channel (Schäfer *et al.*, 2005). Further *cmuA* sequences from soil identified by SIP were similar to that of a marine isolate, strain ''*Roseovarius*'' 179 (Schäfer *et al.*, 2005), and somewhat more distantly related to *cmuA* sequences from the SIP experiments with soil carried out by Miller *et al.* (2004).

The results of this study suggested that *Hyphomicrobium* spp. may be active in particular soils, but also that they are dominant isolates because they are favored by the enrichment and isolation conditions used in these experiments. Other dominant types of MeCl-utilizing bacteria (indicated by direct cloning and sequencing and by SIP experiments) have yet to be cultivated from soils and need to be identified and characterized at the physiological level. The high similarity of *cmuA* sequences from marine enrichments and isolates and certain

cmuA genes detected by DNA-SIP in soils was unexpected and an interesting observation.

C. Marine Methyl Halide Degradation

Relatively little is known about the diversity of methyl halide-degrading bacteria in seawater. Until now, the identification of methyl halide-degrading bacteria has mainly been restricted to isolation of strains that could grow on MeBr or that were able to cometabolize it (Goodwin *et al.*, 1998, 2005; Hoeft *et al.*, 2000; Schaefer *et al.*, 2002; Schäfer *et al.*, 2005). *L. methylohalidivorans* MB2, ''*Roseovarius*'' sp. 179, ''*Roseovarius*'' sp. 217, and ''*Ruegeria*'' sp. 198 were all affiliated with the *Roseobacter* clade (Buchan *et al.*, 2005), but methyl halide utilization is not a general trait of *Roseobacter* group isolates (Schäfer and Murrell, unpublished). Analysis of the diversity of *cmuA* genes obtained by PCR amplification, cloning, and sequencing directly from community DNA extracted from seawater samples and methyl halide-degrading enrichments indicates that additional uncultivated lineages of *cmuA*-containing organisms are present in seawater (Cox, 2005). A report by Goodwin *et al.* (2005) indicates that MeBr may be degraded in the marine environment by bacteria, such as *Sphingomonas* Oxy6, which co-oxidized MeBr using an enzyme that was inhibited by toluene.

V. Potential Applications for Bioremediation Using Methyl Halide-Oxidizing Bacteria

A. Reducing Fugitive MeBr Emissions

Most of the anthropogenic use of MeBr (~50 ktonnes per year) is for biocidal purposes. Of this amount, the majority (70%) is used in shallow fumigation of agricultural soil for control of nematodes, fungi, or weeds prior to planting high-value crops such as tomatoes or strawberries. An additional 25% is used to fumigate commodities, such as nuts or grains, during storage or to eradicate pests during quarantine associated with worldwide shipping of produce or wood products. Only 3% is used for structural fumigation for pest control. Not all of the MeBr applied during these commercial activities is released to the atmosphere, however much of what is released may be controlled by the application of various physical, chemical, or biological barriers. Here we examine the potential use of methylotrophic bacteria, which are emphasized throughout this review, to decrease or eliminate the emission of anthropogenic MeBr to the atmosphere.

B. Previous Efforts to Reduce MeBr Emissions

Before this discussion, it is worth pointing out that other strategies for ameliorating the flux of MeBr to the atmosphere have been proposed and tested with varying results with regard to their overall efficacy and economic practicality. It has long been known that application protocols affect the emission of MeBr during agricultural field fumigations. As much as 90% of the added MeBr may escape to the atmosphere under certain conditions. Injection of fumigant deep in the soil (>30 cm) and optimization of soil moisture content can reduce emissions (Yates *et al.*, 1998), while emplacement of virtually impenetrable film (VIF) over the field during the fumigation period can nearly eliminate losses during the covered period (Wang *et al.*, 1997). Compounds that react rapidly with MeBr (e.g., ammonium thiosulfate) may provide an effective chemical barrier when applied to the soil surface prior to fumigation (Gan *et al.*, 1998b). However, of these mitigation strategies, only deeper injection and premoistening the soil have been adopted as standard practice. Either costs or environmental concerns have, so far, limited the adoption of VIF tarps or chemical flux barriers.

C. Bioremediation Using Methyl Halide-Oxidizing Bacteria

1. *Direct Application of Methylotrophic Bacteria to Soil*

Use of methyl halide-oxidizing methylotrophs to enhance the natural degradation of fumigant MeBr in soils was first proposed by Connell Hancock *et al.* (1998). They observed accelerated degradation of MeBr in soil microcosms inoculated with *A. ciceronei* (then called strain IMB-1). They further showed that IMB-1 could grow on a variety of substrates, including other methyl halides, methyl amines, acetate, and glucose. However, the ability of IMB-1 to oxidize MeBr was constitutive in methyl halide-grown cells but required induction in cells grown on other substrates. Growth on methyl halides was slow and because of the inherent toxicity of these gases, growth could be sustained only if these substrates were added incrementally as discrete pulses to avoid the toxic effects of high single doses. To circumvent this constraint, it proved useful to first grow cells to high density using either glucose or methyl amines and later induce methyl halide oxidation enzymes by exposing them to MeBr (Schaefer and Oremland, 1999).

Practicality of applying bacterial cells directly to surface soils in order to oxidize MeBr was examined during field fumigation of strawberry fields in Monterey, CA. Cells of *A. ciceronei* suspended in minimal phosphate media (3×10^{12} cells m^{-2}) were applied to the soil

surface using a garden sprayer immediately prior to fumigation. A mixture of MeBr and the warning agent and biocide chloropicrin (CCl_3NO_2), in a ratio of 67% to 33% w/w, was injected by a tractor moving along the surface. A 0.025-mm thick polyethylene sheet was simultaneously rolled over the surface and covered the field. MeBr concentrations in the soil were determined during the first 4 days of fumigation at three depths below and one depth above the soil surface beneath the plastic sheet (Fig. 5). Following the 6-day fumigation period, the sheets were removed and accumulated Br^-, the product of both biological and chemical mineralization of MeBr, was extracted from the soil using deionized water (Fig. 6). More MeBr was mineralized in soils that received media (with or without cells) than in the controls, indicating that some mineralization was due to nucleophilic reaction of MeBr with water (hydrolysis) or dissolved salts (halide exchange) in the media. A greater amount of MeBr was mineralized in soils that received media plus *A. ciceronei*, and this additional amount was attributed to oxidation by the bacteria.

Enhanced mineralization of MeBr resulting from addition of media was ubiquitous (data not shown); however, addition of bacterial cells did not always stimulate oxidation. Several factors may have caused an inhibition of the desired bacterial activity, including (1) locally elevated concentrations of MeBr (>5%) beneath the plastic sheet, resulting in direct toxicity to the added *A. ciceronei* cells, (2) elevated temperatures (>40°C) beneath the plastic sheet, exceeding the limits of *A. ciceronei*,

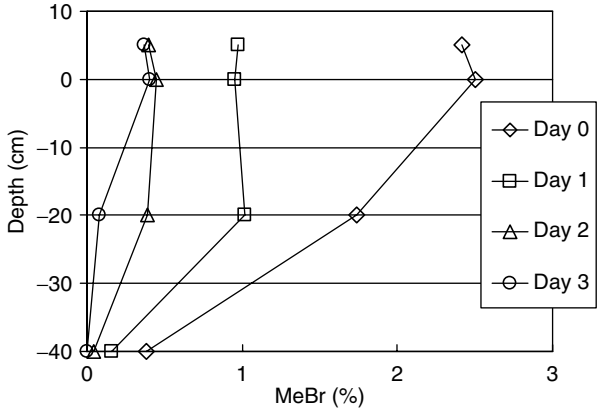

FIG. 5. MeBr concentrations in soil gas during the first 4 days of field fumigation with MeBr (67%) and chloropicrin (33%). MeBr was added around 20 cm depth on day 0 at a rate of 160 kg/acre.

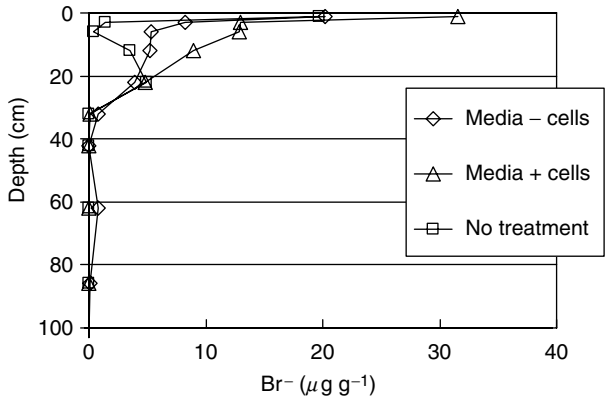

FIG. 6. Bromide ion (Br^-) extracted from soil fumigated with MeBr and chloropicrin (6 days) collected under various soil surface treatments.

and (3) high concentrations of chloropicrin, a known inhibitor of MeBr oxidation by *A. ciceronei* (Connell Hancock *et al.*, 1998). These three variables were deemed difficult to control during commercial fumigations, hence direct application of methylotrophic bacteria for the purpose of providing a biological barrier to MeBr flux was abandoned, with the recommendation that if and when these variables could be controlled, application of IMB-1 to the soil surface could greatly reduce MeBr emission during field fumigations.

2. *Using Bacteria to Remove MeBr from the Waste Stream of Contained Fumigations*

Several nonbiological strategies that use ammonium thiosulfate to scrub MeBr emitted from field fumigations have been proposed. Chitwood and Deshusses (2001) envisioned collection of MeBr between two layers of plastic sheet during field fumigations and transport by fans to reaction chambers containing a chemical scrubber. Value Recovery, Inc., a New Jersey company, is testing a version of this idea using an additional proprietary catalyst in the scrubber. Other proposed strategies rely on physical trapping of MeBr using activated charcoal or zeolite materials but these methods store MeBr and do not destroy it. They may be useful when chemical or biological destruction can be achieved off site at some convenience.

Some of the above mentioned nonbiological strategies have been suggested or are already in use to remove MeBr from quarantine and pre-shipment (QPS) fumigations (Gan and Yates, 1998). Physical traps

(e.g., activated charcoal) require further treatment (usually heating) to release MeBr for recycling or destruction. These and the chemical scrubbers used commercially to treat QPS fumigations are subject to breakthrough of MeBr once the number of active sites or the kinetics of the aqueous-phase reaction is exceeded. In addition, they have difficulty complying with proposed USEPA regulations limiting maximum emissions to <500-ppm MeBr during operation. Most of these problems can be overcome using bioreactors which utilize the activity of MeBr-oxidizing bacteria to completely eliminate emissions from QPS fumigations (Miller *et al.*, 2003). Products of laboratory bioreactors using *A. ciceronei* were CO_2 and Br^-, and the reactors functioned at 100% efficiency for long periods of time when pH and temperature were controlled. Scaling up the laboratory bioreactors in order to demonstrate the economic use of bacteria to destroy MeBr in QPS fumigations presents simple engineering challenges but as of this writing has not yet been attempted.

VI. Outlook

A. Genomics of Methyl Halide-Degrading Bacteria

The study of methyl halide degradation pathways will further benefit from the genome-sequencing projects for *M. chloromethanicum* CM4 and *Roseovarius* sp. 217. In the latter, methyl halide degradation occurs by an as yet uncharacterized pathway. Preliminary analysis of the genome sequence indicates the presence of a number of enzymes involved in C_1 metabolic pathways, for example certain enzymes of the serine cycle of formaldehyde assimilation (Schäfer and Murrell, unpublished). The genome sequence does not contain *cmuA*, thus confirming results of earlier Southern hybridization and PCR analyses. Mass spectrometry of proteins induced during growth on methyl halides is currently being carried out to identify candidate genes that could play a role in metabolism of methyl halides (Schäfer and Murrell, unpublished).

B. Contribution of Alternative Pathways of Methyl Halide Degradation

One of the future challenges will be to assess the contribution of the various pathways of methyl halide degradation to the overall biological sink. The work by Goodwin *et al.* (2005) demonstrates that toluene is a potent inhibitor of MeBr oxidation in the marine environment. It remains to be assessed whether toluene may also be an inhibitor of

methyl halide oxidation in extant marine methyl halide-degrading isolates other than *L. methylohalidivorans*, which was not inhibited by toluene. Degradation of methyl halides by bacteria is most probably carried out by a number of different pathways. A better understanding of bacterial cycling of methyl halides will require additional research to characterize the unknown methyl halide degradation pathways, for instance in *Roseovarius* sp. 217 and *L. methylohalidivorans*. While the contribution of individual pathways will only be assessable once suitable inhibitors have been identified, including those that inhibit the CmuA pathway, functional gene markers will need to be developed in order to address the functional diversity of methyl halide-utilizing bacteria that do not employ the CmuA pathway of methyl halide degradation. Metabolomics studies using methyl halides labeled with stable or radioactive isotopes coupled to nuclear magnetic resonance analysis might offer a chance to follow the fate of the methyl carbon in order to assess the dominant assimilation pathways for methyl halides.

C. Evolutionary Aspects and Role of Methyl Halides as Substrates in the Environment

The detection of closely related *cmuA* sequences in forest soils and marine isolates is intriguing. At least in some of the marine isolates, there is currently no evidence for additional enzymes of primary methylotrophic metabolism since thus far, the methyl halides are the only C_1 substrates that support growth of strains ''*Rosovarius*'' sp. 179 and ''*Ruegeria*'' sp. 198, out of the wide range that have been tested (Schäfer, unpublished data). Future genome sequencing of methyl halide-utilizing isolates might help to answer the question whether methyl halide degradation pathways first evolved in the terrestrial or the marine environment. The fact that a number of the known genes involved in methyl halide degradation are evolutionary related to genes encoding enzymes of C_1 metabolism in methanogenic archaea further underlines the possibility that horizontal gene transfer may have played an important role in the diversification of C_1 compound metabolism and the spread of methyl halide utilization genes.

Acquisition and retention of genes for methyl halide utilization probably confers a selective advantage to organisms in environments where methyl halides are produced (e.g., soils, the phycosphere). It has been suggested previously that it is unlikely that methyl halide-degrading organisms could live on atmospheric levels of MeBr (Hoeft *et al.*, 2000), but since tropospheric levels of MeBr were readily

consumed by *A. ciceronei* IMB-1 and *L. methylohalidivorans* MB2, it is likely that methyl halides can be exploited by facultative methylotrophs as supplementary carbon sources in both soil and marine environments. Bacterial consumption of methyl halides would thus supply extra energy (and could potentially also lead to carbon assimilation) while the cell is using other growth substrates (Goodwin *et al.*, 2001). This does not rule out the possibility of microsites in the environment that might have higher concentrations of methyl halides that could potentially support growth. Such microsites might exist in soils in proximity of methyl halide-producing fungi and plants, or in the marine environment in the phycosphere of phytoplankton producing MeBr.

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