ADVANCES IN
APPLIED MICROBIOLOGY

VOLUME 49

ACADEMIC PRESS

ADVANCES IN APPLIED MICROBIOLOGY

Volume 49

Allen I. Laskin

ADVANCES IN

Applied Microbiology

VOLUME 49

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ADVANCES IN

Applied Microbiology

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VOLUME 49

ACADEMIC PRESS

San Diego New York Boston
London Sydney Tokyo Toronto

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Academic Press
A division of Harcourt, Inc.
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http://www.academicpress.com

Academic Press
Harcourt Place, 32 Jamestown Road, London NW1 7BY, UK
http://www.academicpress.com

International Standard Serial Number: 0065-2164

International Standard Book Number: 0-12-002649-X

PRINTED IN THE UNITED STATES OF AMERICA
01 02 03 04 05 06 MM 9 8 7 6 5 4 3 2 1

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I. Introduction

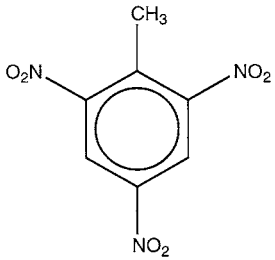
The contamination of the environment with explosive residues presents a widespread environmental problem. Military sites of production, processing, and disposal of these explosives are the main concern with the manufacture of explosives representing a sizable segment of the chemical industry. The relative recalcitrance of many of these compounds means that levels of contamination can build up over many years, with sites used in World War II still heavily contaminated. As these compounds are toxic, mutagenic, and highly energetic (Rosenblatt *et al.,* 1991), they present a serious problem.

The explosives of the greatest importance are the high explosives: nitroaromatics [such as 2,4,6-trinitrotoluene (TNT) and picric acid],

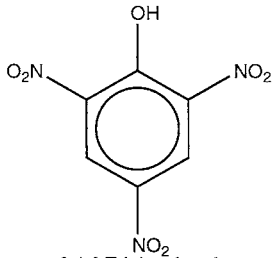
ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 49

0065-2164/01 $35.00

NITROAROMATICS

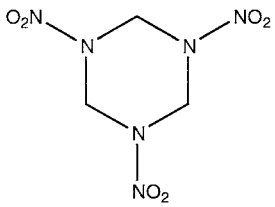


2,4,6-Trinitrotoluene
(TNT)

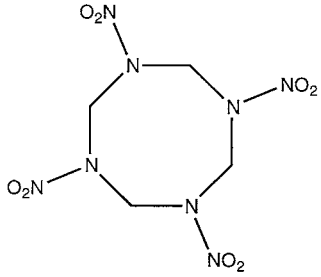


2,4,6-Trinitrophenol
(PICRIC ACID)

NITRAMINES

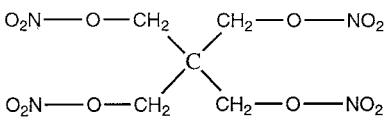


Hexahydro-1,3,5-trinitro-1,3,5-triazine
(RDX)

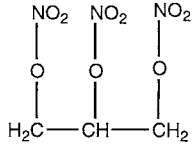


Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
(HMX)

NITRATE ESTERS



Pentaerythritoltetranitrate
(PETN)



Glyceroltrinitrate
(GTN)

FIG. 1. Molecular structures of the most important high explosives.

nitramines [Royal Demolition Explosive—hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX)—and High Melting Explosive—octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX)], and nitrate esters [pentaerythritol tetranitrate (PETN) and glycerol trinitrate (GTN)]. Figure 1 shows the structures of these compounds. The high proportion of nitro groups is an important property of explosives. TNT is historically the most important explosive; however, heterocyclic nitramines have now taken over as the most widely used military explosives.

During the production of explosives large quantities of water are required due to the insolubility of the compounds. This has resulted in the siting of manufacturing plants near large supplies of groundwater, subsequently used for drinking water reservoirs (Levsen *et al.,* 1993). The vast quantities of water involved leach through soil and eventually into ground water again, as well as into surface water, carrying and depositing explosive residues (Kaplan and Kaplan, 1982). Soil is also contaminated in the disposal of substandard or out-of-date munitions. Open burning or incineration are the commonest forms of disposal, although landfill sites and lagoons, which have been used for dumping, still exist. Particularly high concentrations of explosives have been detected on lagoon beds. Again contamination of ground and surface water through seepage is a problem.

The persistence of the explosives in the environment ensures that the problem escalates with time. As environmental awareness has and continues to increase, it has become necessary to find alternative means of disposal, as well as of eradicating the existing problem. A potential solution to the environmental contamination by explosives is bioremediation. This offers a cheap and environmentally friendly alternative to the harsher and more expensive alternatives of chemical or physical degradation.

Research into the breakdown of explosives has resulted in the isolation of organisms with biodegradative capabilities. The pathways by which this occurs have been elucidated and some of the enzymes responsible extracted in their active form. This knowledge will be of significant use in the design of biological remediation systems to clean up areas contaminated with these energetic materials.

II. Nitroaromatic Explosives

A. The Chemical Nature of TNT and Its Reactivity

The electronic nature of TNT governs the type of reactions the explosive can undergo in biological systems. The π electrons of the aromatic ring system are withdrawn by the nitro groups, making the nucleus

electron deficient and resistant to electrophilic attack. This has a profound effect on the mechanisms by which TNT can be transformed in the environment. Degradation of aromatic pollutants by aerobic bacteria usually occurs initially by electrophilic attack by oxygenases on the ring system (Spain, 1995a, 1995b). This also occurs to a certain degree with mononitroaromatic and dinitroaromatic compounds. But with increasing number of nitro substitutions, the ring systems becomes more resistant to attack by oxygenases. This is supported by the observation that oxygenase reactions are unknown for trinitro compounds such as TNT or picric acid. Instead, the nitro groups are extremely susceptible to reduction; this proceeds via the nitroso group (rarely isolated) and the hydroxylamino group to the amine. Hydroxylamines and amines are also reactive species, and can form a wide range of covalent adducts. For example, during the reduction process, a hydroxylaminodinitroaromatic compound may attack a nitrosodinitroaromatic compound to form a tetranitro-azoxy dimer. It appears that these reduced intermediates may also attack a variety of biological molecules to form covalent attachments, thus exerting their toxic effects (see Sec. II.B).

The electron deficiency of the aromatic ring means that TNT is also highly susceptible to attack by nucleophiles to form Meisenheimer complexes (sigma complexes) (Buncel, 1982; Crampton, 1969). These complexes are generally negatively charged and often brightly colored due to an extensive delocalized electron system. If the attacking species is a hydride ion, a hydride–Meisenheimer complex will be formed (Kaplan and Siedle, 1971; Buncel, 1982). A second hydride ion may attack the hydride–Meisenheimer complex to give a dihydride–Meisenheimer complex. In the case of TNT, initial hydride attack typically occurs at position 3. Recent evidence (see Sec. II.B) suggests that hydride–Meisenheimer complexes may play an important role in the productive degradation of TNT and picric acid in some bacteria.

In biological systems the most common transformation of TNT is via reduction of the nitro groups. The formation of hydroxylamino derivatives is of greatest concern as these compounds readily modify DNA and thus introduce mutations. This type of activation is responsible for the toxicity and mutagenicity of many nitroaromatic compounds (Bryant and McElroy, 1991). Thus the initial effects of biological systems is to inadvertently activate nitroaromatic compounds to produce metabolites which have significantly higher toxicity than the original chemical. Reduction of the nitro group is usually an accidental side reaction catalyzed by a wide variety of enzymes possessing redox-active flavins or metal ions, which are collectively referred to as nitroreductases. In the great majority of cases, reduction of aromatic nitro groups is not their

primary physiological function, although nitroreductases have been shown to occur as part of the microbial degradation pathways for mononitroaromatic compounds such as nitrobenzoate (Chauhan and Jain, 2000; Ybannavar and Zylstra, 1995; Groenewegen *et al.,* 1992), nitrobenzene (Park and Kim, 2000; Peres *et al.,* 1998), nitrophenol (Schenzle *et al.,* 1997; Blasco and Castillo, 1993), and chloronitrophenol (Schenzle *et al.,* 1999), in which degradation proceeds after reduction of the aromatic nitro group to the hydroxylamine or amine. Reduced TNT metabolites become irreversibly bound to soil components following biological reduction (Daun *et al.,* 1998; Lenke *et al.,* 1998). Studies using [^{14}C]TNT have demonstrated that reduced TNT derivatives become incorporated into humic and fulvic acids (Pennington *et al.,* 1995; Hundal *et al.,* 1997; Achtnich *et al.,* 1999a; Drzyzga *et al.,* 1998a, 1998b). The nature of the condensation products has been studied using [^{15}N]TNT together with ^{15}N-NMR (nuclear magnetic resonance) spectrometry (Knicker *et al.,* 1999; Achtnich *et al.,* 1999b; Bruns-Nagel *et al.,* 2000). The remaining nitro groups in TNT derivatives, which have become incorporated into macromolecules, also act as substrates for microbial reduction, although the reaction rates are slower than those seen for nitro groups on free nitro compounds (Achtnich *et al.,* 1999c).

Over the past 10 years numerous reviews have been written on the ability of pure and mixed cultures to transform TNT and other nitroaromatic compounds (Higson, 1992, Marvin-Sikkima and de Bont, 1994; Crawford, 1995; Lewis *et al.,* 1995; Spain, 1995a, 1995b; Preuss and Rieger, 1995; Rieger and Knackmuss, 1995; Funk *et al.,* 1996 and Nishino *et al.,* 2000). Research into the microbial transformation and degradation of TNT has shown that the products formed are dependent on the growth conditions and the species involved. The recalcitrant nature of TNT means that enzymes from a diverse range of microbes and growth conditions may be required for the total remediation of contaminated soil.

B. Aerobic Transformation of TNT by Bacteria

A review of the literature shows that a number of different species are able to transform TNT. But one genus appears with a greater frequency than all others, which is the *Pseudomonas* species. This probably reflects the ease by which these organisms can be cultured from contaminated soil samples in the laboratory. Many other species may exist that have the ability to transform TNT, but these may be elusive due to specialized nutrients requirements or growth conditions needed

for culturing. Standard batch culture enrichment techniques typically select for rapidly growing microbes; thus slow growing organisms will be excluded over time. Also, enrichment procedures which select for microbes which degrade recalcitrant compounds may take several months to grow. This may be because the compounds that are used as energy sources are metabolized inefficiently, toxic at high concentrations, or only poorly soluble (Dunbar *et al.,* 1996).

Whole cell incubations of a number of bacterial species and TNT have revealed that many aerobic bacteria possess enzymes with nitroreductase activity. The majority of the products identified are reduced products of TNT such as aminodinitrotoluenes (unspecified mixture of 2-amino-4,6-dinitrotoluene and 4-amino-2,6-dinitrotolune) (ADNT) or diaminonitrotoluenes (unspecified mixture of 2,4-diamino-6-nitrotoluene and 2,6-diamino-4-nitrotoluene) (DANT), but typically no further reduced species are observed. For example, several *Pseudomonas* strains have been shown to reduce TNT to ADNT and DANT (Schackmann and Muller, 1991) (Fig. 2). An NAD(P)H-dependent enzyme (NADH: reduced nicotinamide adenine dinucleotide; NADPH: reduced nicotinamide adenine dinucleotide phosphate) in crude extracts was found to catalyze these reductive processes; this was presumably an oxygen-insensitive nitroreductase.

Pseudomonas fluorescens has been reported to be able to reduce TNT to ADNT and then to 24DA6NT and 26DA4NT (Gilcrease and Murphy, 1995). But unusually, 2,4-diamino-6-nitrotoluene (24DA6NT) was then acetylated to 4-N-acetylamino-2,6-dinitrotoluene. Neither 2,6-diamino-4-nitrotoluene (26DA4NT) or 4-N-acetylamino-2,6-dinitrotoluene was further degraded and accumulated in the culture media.

The degradation of the reduced products of TNT has been observed with some aerobic bacteria. For example, a consortium of several *Pseudomonas* spp. was able to reduce TNT to ADNT. Further incubation

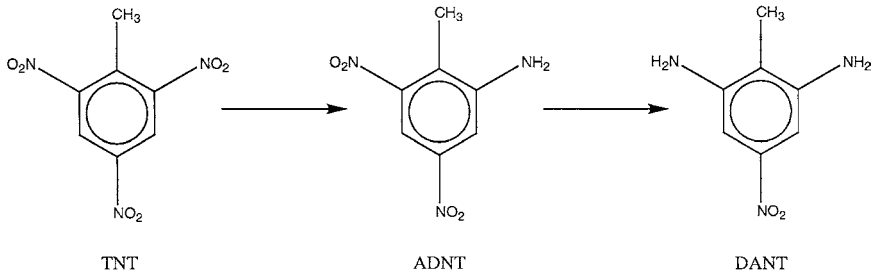


FIG. 2. Transformation of TNT under aerobic conditions by several *Pseudomonas* strains.

in the presence of succinate resulted in the incorporation of a small amount of [^{14}C]TNT into biomass, and the release of some radioactivity as $^{14}CO_2$ (Boopathy *et al.,* 1994a). From this consortium, four *Pseudomonas* spp. were isolated—all of which were able to transform TNT to give the most significant product as ADNT, as observed in the original mixed culture (Boopathy *et al.,* 1994b). However, nitrite was also observed, with levels of up to 1 mol per mol TNT. Up to 13% of the ^{14}C from the TNT was incorporated into biomass after prolonged incubation.

Pseudomonas aeruginosa MAO1 was originally isolated for its ability to use athranilate (2-aminobenzoate) as a sole carbon source, but subsequently it was shown to reduce TNT to ADNT in the presence of succinate. Any further degradation of the ADNT was dependent on oxygen, which suggests a role for an oxygenase to generate unidentified polar products (Alvarez *et al.,* 1995). Approximately 45% of the radioactivity from [^{14}C]TNT appeared in polar products and DANT was not produced. It was shown that when the organism was incubated with 24DA6NT, the compound was N-acetylated, and not further metabolized.

Fiorella and Spain (1997) reported a novel transformation pathway for TNT in *Pseudomonas pseudoalcaligenes* JS52. Nitrobenzene was used as a sole carbon source and cell-free extracts were found to transform TNT to 4-hydroxylamino-2,6-dinitrotolune (4HA26DNT), 4-amino-2,6-dinitrotoluene (4A26DNT), 2,4-dihydroxylamino-6-nitrotoluene, and 4-amino-2-hydroxylamino-6-nitrotoluene, in the presence of NADPH (Fig. 3). Transformation of TNT by whole cells only occurred if nitrobenzene was present, indicating that TNT transformation did not provide energy for bacterial growth. Also, experiments with [^{14}C]TNT indicated that the explosive itself was not mineralized, although ^{14}C was found to be associated with the cells. The authors also showed that TNT was reduced to DHANT via 4HA26DNT by the enzyme nitrobenzene nitroreductase, which had been purified from *P. pseudoalcaligenes* JS52.

Isolation of actinomycetes from TNT-contaminated or- uncontaminated sites showed that these species were able to reduce TNT, but did not mineralize it to a significant degree (Pasti-Grigsby *et al.,* 1996). Little difference was seen between isolates from contaminated environments and those from uncontaminated environments, consistent with the view that these transformations are essentially fortuitous and involve widely distributed enzymes.

Reduction of the nitro groups is not the only chemical transformation of TNT that has been observed. Vanderberg *et al.* (1995) reported that a strain of *Mycobacterium vaccae,* when grown with propane as sole carbon source, generated a variety of oxidized products, including

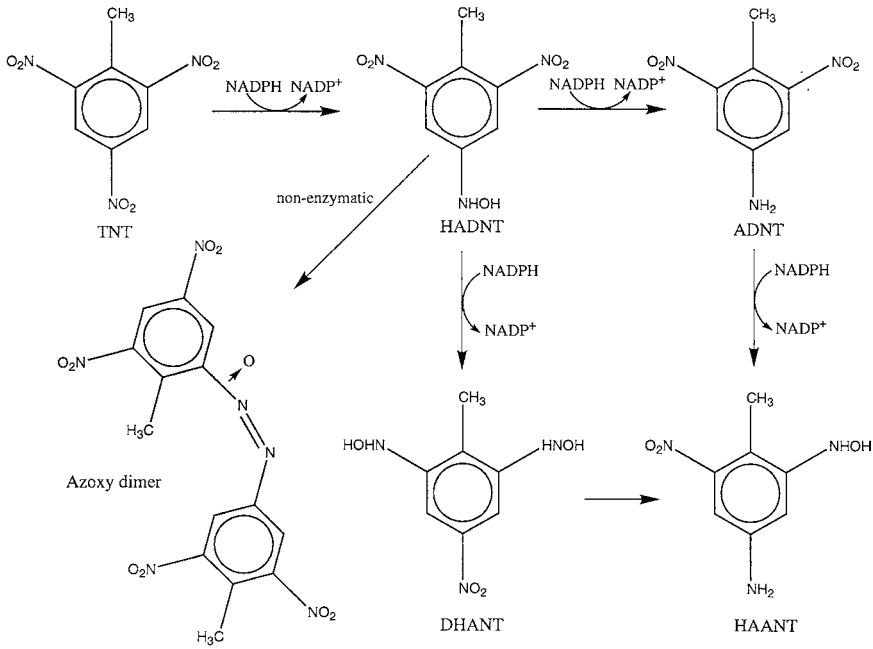


FIG. 3. Transformation of TNT by nitrobenzene nitroreductase purified from *Pseudomonas pseudoalcaligenes* JS52. DHANT: dihydroxylaminonitrotoluenes (unspecified mixture of 2,4-dihydroxylamino-6-nitrotoluene and 2,6-dihydroxylamino-4-nitrotoluene); HAANT: hydroxylamino-aminonitrotoluene.

4-amino-2,6-dinitrobenzoic acid. Thus the methyl group of TNT had been oxidized as well as the reduction of the 4-nitro group. It was speculated that the oxidation of the methyl group was carried out by a propane monooxygenase which had a low substrate specificity. Incubation with [^{14}C]-TNT and propane resulted in the incorporation of approximately half of the radioactivity in cellular lipids, suggesting that ring cleavage might have occurred.

A number of other pathways exist by which TNT can be transformed. Apart from the reductive pathway, TNT has been shown to undergo a range of transformations including denitration and hydride addition to the aromatic ring system.

TNT has been reported to undergo denitration to give DNT and nitrite. Martin *et al.* (1997) identified a *Pseudomonas savastanoi* strain which had been isolated from contaminated soil from a Nebraska Ordnance Plant. This isolate was able to denitrate TNT to give 2,4-dinitrotoluene and nitrite. Typical nitroreductase products, ADNT, were also produced, particularly in the presence of glucose; denitration of TNT was favored

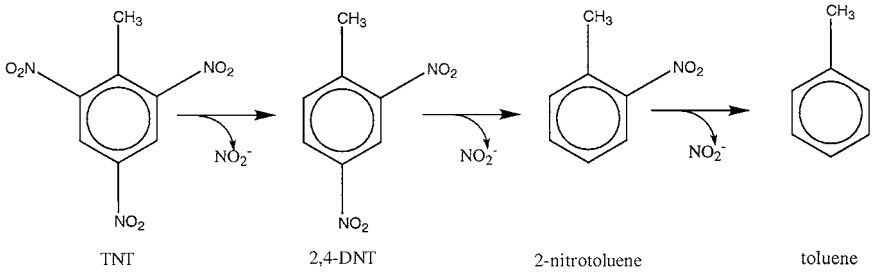


FIG. 4. Denitration of TNT by *Pseudomonas* sp. clone A.

by absence of ammonium and presence of nitrite. There was no significant production of $^{14}CO_2$ from labeled TNT.

Duque *et al.* (1993) isolated a *Pseudomonas* strain C1S1 which was able to grow on TNT or dinitrotoluenes as a sole source of nitrogen, but required an additional carbon source. Nitrite was seen to accumulate in the medium. Batch culture enrichment was used to isolate a derivative strain, called *Pseudomonas* sp. clone A, which grew faster on TNT and did not accumulate nitrite in the culture medium. It was shown that the both strains denitrated TNT to give 2,4- and 2,6-dinitrotoluene, 2-nitrotoluene, and toluene (Fig. 4). The TOL plasmid was introduced into *Pseudomonas* sp. clone A using conjugation, and the resultant bacteria was able to grow on TNT as the sole nitrogen and carbon source. The hydride–Meisenheimer complex of TNT was identified in culture supernatants, and cell extracts were reported to transform the chemically synthesized hydride–Meisenheimer complex to 2,4-dinitrotoluene and another unidentified product, possibly 1,3,5-trinitroheptane or 4-methyl-1,3,5-trinitrohexane (Haïdour and Ramos, 1996). *Pseudomonas* sp. strain A also produced typical nitroreductase products from TNT, including tetranitro-azoxy dimers. These accumulated in the culture medium and appeared to be dead-end products, presumably arising through fortuitous reduction of TNT by various redox enzymes. The proposed pathway for the denitration of TNT to dinitrotoluenes (unspecified mixture of dinitrotoluene and 2,6-dinitrotoluene) (DNT) was suggested to proceed in an analogous manner to that observed with picric acid, i.e., reductive denitration, hydride addition with subsequent nitrite elimination (Lenke and Knackmuss, 1992). The Gram-positive strain *Rhodococcus erythropolis* HL PM-1 aerobically transformed picric acid by the addition of hydride at the 3 postion of the compound to form a hydride–Meisenheimer complex. Elimination of nitrite from the intermediate was observed, and the aromaticity was reformed to give 2,4-dinitrophenol (Lenke and Knackmuss, 1992). However, the

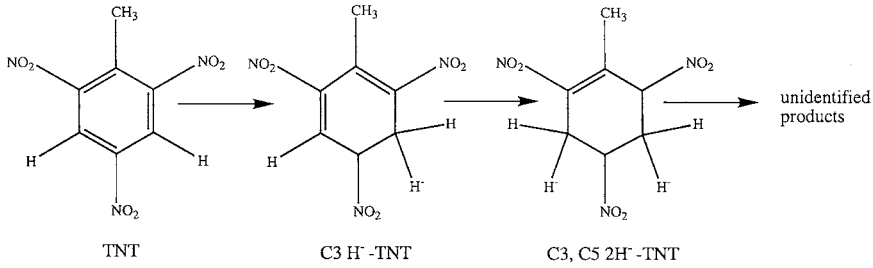


FIG. 5. Ring reduction of TNT by hydride addition. Initial reduction at C3 gives hydride–Meisenheimer complex (H^--TNT). Subsequent addition at C5 gives the dihydride–Meisenheimer complex ($2H^-$-TNT).

proposed pathway for TNT degradation by *Pseudomonas* sp. A was later questioned by Vorbeck *et al.* (1998). It was reported that this organism, and another Gram-negative organism, were able to grow on TNT as a sole nitrogen source in the presence of a suitable carbon source (Vorbeck *et al.,* 1998). TNT was transformed to the hydride–Meisenheimer intermediate, but no dinitrotoluenes or elimination of nitrite was observed. Instead, the usual reduced metabolites of TNT transformation were seen [hydroxylaminodinitrotoluenes—unspecified mixture of 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene (HADNTs and ADNTs)]. The possible source of nitrogen for growth was speculated to originate from HADNT to give unidentified polar products.

A significant number of organisms initially isolated for their ability to transform other nitro-containing compounds have subsequently been shown to transform TNT. A common observation is the formation of the hydride or dihydride–Meisenheimer complex with the release of nitrite (Fig. 5). For example Vorbeck *et al.* (1994), isolated a strain of *Mycobacterium* sp., designated HL-4-NT-1, for its ability to grow on 4-nitrotoluene as a sole source of nitrogen. It was also shown that this microbe transformed TNT to produce a red, water-soluble metabolite, which was identified as the hydride–Meisenheimer. The release of nitrite was observed, but no DNT was detected. Both HL-4-NT-1 and *R. erythropolis* HLPM-1 could further reduce the hydride–Meisenheimer complex to form the dihydride–Meisenheimer complex (Vorbeck *et al.,* 1998).

Enterobacter cloacae PB2 was isolated for its ability to grow on the nitrate esters GTN and PETN as sole nitrogen sources, but it was also shown that *E. cloacae* PB2 would grow very slowly on TNT as a sole nitrogen source (French *et al.,* 1998). It was found that the enzyme responsible for the denitration of the nitrate esters, PETN reductase, could

also reduce TNT in an NADH-dependent reaction. From the analysis of the products formed from the reduction of TNT, it appeared that PETN reductase had the ability to reduce both the nitro groups and the aromatic ring of TNT. Thus PETN reductase reduced TNT to give HADNT and ADNT, as observed with other nitroreductases, but also had the ability to channel TNT down the hydride ring addition pathway. The products of the hydride pathway were identified as the hydride and dihydride–Meisenheimer complexes. Furthermore, the dihydride–Meisenheimer complex appeared to undergo further degradation, either spontaneous or catalyzed at a low rate by PETN reductase, to give 1 mole of nitrite per mole of TNT. A range of other unidentified, water-soluble, nonaromatic products were also detected. The precise nature of the end products of the hydride pathway are as yet still unknown. The nitrite liberated from TNT is most likely to have originated from the hydride addition pathway than from the direct reduction of the nitro groups, as nitrite is not liberated from TNT in the presence of other oxygen-insensitive nitroreductases. Recently Pak *et al.* (2000) examined the enzymatic transformation of TNT by purified XenB, an NADH-dependent flavoprotein oxidoreductase from *Pseudomonas fluorescens* I-C; like PETN reductase XenB reduced TNT to hydride–Meisenheimer complexes and 2-hydroxylamino-4,6-dinitrotoluene and 4-hydroxylamino-2,6-dinitrotoluene. Furthermore, Pak *et al.* (2000) went on to show that the products of aromatic ring reduction and nitro group reduction underwent nonenzymatic dimerization resulting in the formation of isomers of amino-dimethyl-tetranitrobiphenyl, which may account for the observed nitrite release (Fig. 6). In contrast to the results reported by Haïdour and Ramos (1996), none of these reports described above show the formation of DNT from the breakdown of the hydride–Meisenheimer complex.

The scope for complete mineralization of TNT by the aerobic systems discussed above may be of limited use. The formation of dead-end metabolites excludes their use as possible sources of energy, and so transformation does not proceed fully to mineralization. Also, these products, including highly reactive species such as HADNT and ADNT which are actually more toxic than TNT itself, cause the chemical modification of proteins, lipids, and carbohydrates. This would have an adverse effect on any biological system which may utilize TNT for growth and therefore limits total mineralization.

C. Anaerobic Transformation of TNT by Bacteria

Microbial degradation of organic matter and respiration within the soil brings about the formation of anaerobic microenvironments. As a

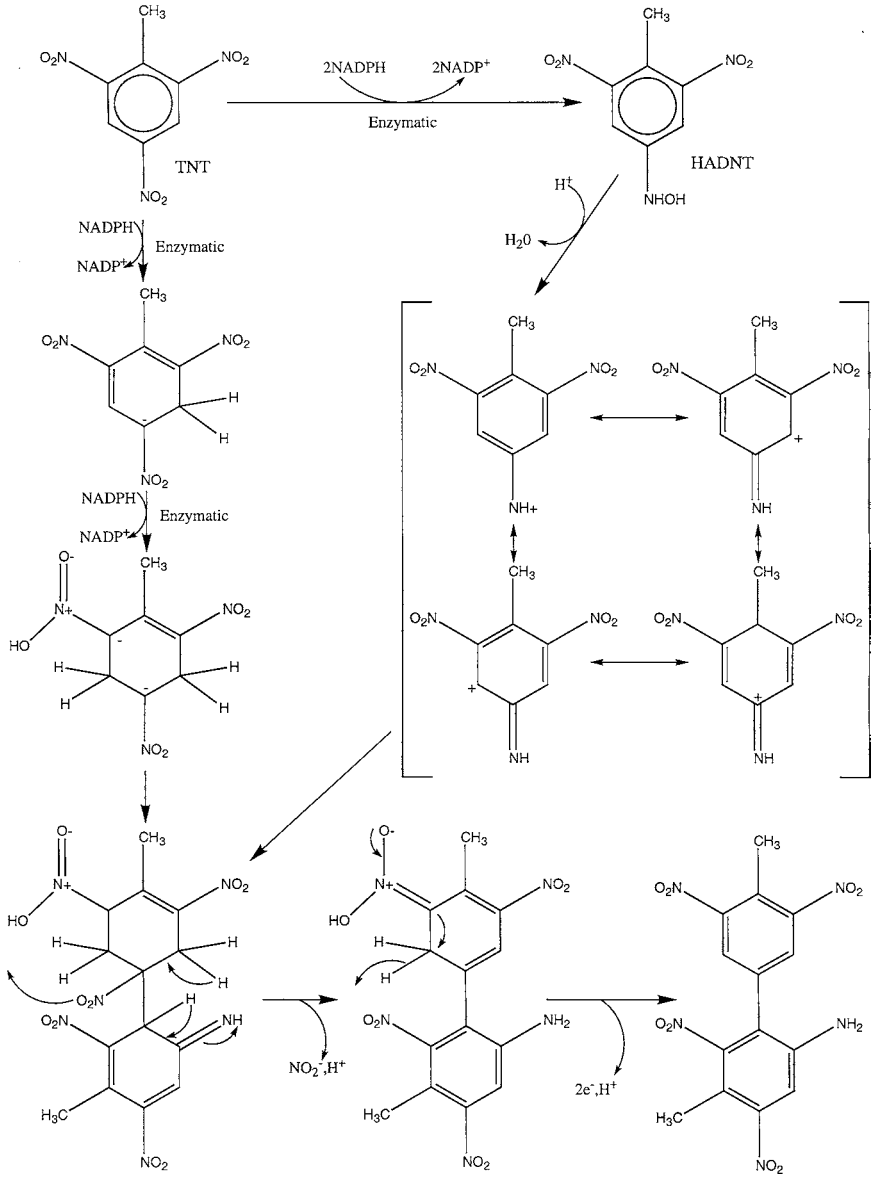


FIG. 6. Transformation of TNT by purified XenB from *Pseudomonas fluorescens* I-C. Both enzymatic and nonenzymatic steps are shown.

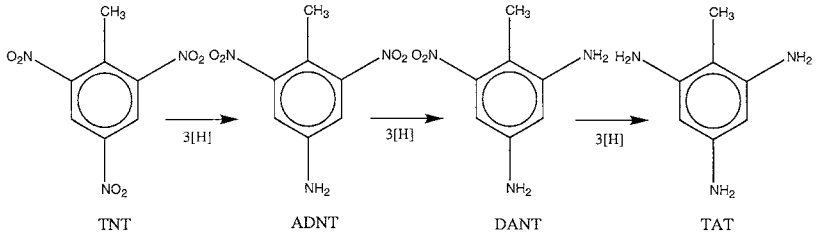


FIG. 7. Anaerobic transformation of TNT.

result, aerobic and anaerobic microenvironments can coexist within and around soil particles. Therefore it is important to consider both environments when looking for organisms that can transform TNT. Reduction of TNT under anaerobic conditions is generally more extensive than that seen under aerobic conditions. Due to the electronegative nature of the nitro group, TNT is more susceptible to reductive, rather than oxidative, attack. Formation of the amino substitute weakens the original electron deficiency present in the ring system of TNT and so the rate of subsequent reduction of the remaining nitro groups is reduced. As a consequence of this, formation of TAT from TNT requires strict anaerobic conditions. Under these strong reducing conditions, all the nitro groups of TNT can be fully reduced to TAT (Fig. 7). Degradation of explosives under anaerobic conditions has been reviewed by Boopathy *et al.* (1998b), Crawford (1995), and Preuss and Rieger (1995).

D. TRANSFORMATION OF TNT BY METHANOGENS AND FERMENTATIVE BACTERIA

The number of identified organisms which reduce TNT under anaerobic conditions is limited. A methanogenic bacteria, resembling *Methanococcus,* was identified by Boopathy and Kulpa (1994) with the ability to reduce TNT to 24DA6NT when provided with formate or carbon dioxide and hydrogen for methanogenesis. This is the only reported case to date of a methanogen being able to transform TNT, although several other strains had been noted to transform other nitroaromatic compounds (Boopathy, 1994).

The fermentative bacteria belonging to the genus *Clostridium* have also been identified as being able to transform TNT. Clostridia are Gram-positive obligate anaerobes, which are found in soil, lake sediments, and intestinal tracks of various animals. Regan and Crawford (1994) reported that a strain of *Clostridium bifermentans* was able to reduce both TNT and RDX. Further studies by Lewis *et al.* (1996) showed that the main metabolites of TNT reduction were 2,4,6-triaminotoluenes

(TAT), phenolic products apparently derived from TAT hydrolysis, and an adduct of TAT formed by condensation with methyl glyoxal (pyruvic aldehyde). TAT readily forms covalent adducts with humic substances in soil and becomes irreversibly bound to the matrix. Therefore this was considered to be a potentially useful route for the bioremediation of TNT-contaminated soil, and so methods for the production of *C. bifermentans* spores, to be used as inocula, were developed (Sembries and Crawford, 1997).

Ederer *et al.* (1997) tested several bacterial strains for their ability to degrade TNT. The strains examined included various clostridial strains isolated from a 4-year-old munition enrichment and related clostridial strains obtained from a culture collection. All of the *Clostridium* species tested were able to reduce TNT to DANT, and then to TAT and several unidentified products. Thus a common pathway for the reduction of TNT must exist within these microbes irrespective of whether they had been previously exposed to the explosive. Khan *et al.* (1997) also reported that *Clostridium acetobutylicum* cells could reduce TNT to water soluble, but undetermined, end products via monohydroxylamino derivatives. The study of this organism and its transformation of TNT was continued by Hughes *et al.* (1998a, 1998b). It was demonstrated that 2-amino-4-hydroxylamino-5-hydroxy-6-nitrotoluene was formed from TNT by cells, and cell extracts, in the presence of H_2 via rearrangement of 2,4-dihydroxylamino-6-nitrotoluene. Dinitrotoluenes were also reduced mainly to dihydroxylaminotoluenes (Hughes *et al.,* 1999), although rearrangements were not observed.

E. Sulfate-Reducing Bacteria

Methanogenic and fermentative bacterial systems will reductively transform TNT; however, sulfate-reducing bacteria have been shown to utilize TNT as a sole nitrogen source (Boopathy *et al.,* 1998a). Sulfate-reducing bacteria are all obligate anaerobes and occupy numerous terrestrial and aquatic environments that become anoxic as a result of microbial degradation process.

A *Desulfovibrio* strain was isolated by Boopathy and Kulpa (1992), which utilized TNT as a sole nitrogen source or as a terminal electron acceptor for respiration. TNT was seen to be degraded to toluene via DANT under nitrogen limiting conditions. It was assumed that the pathway proceeded via TAT, although TAT was not detected. Conversion to toluene occurred only in the absence of ammonium. A variety of other nitroaromatic compounds could also be used as terminal electron acceptors or sources of nitrogen (Boopathy and Kulpa, 1993). Further characterization of this strain suggested that it might be useful, in conjunction with

a toluene-degrading organism, for bioremediation of TNT-contaminated soil and water under anaerobic conditions (Boopathy *et al.,* 1993).

A sulfate-reducing bacterium using TNT as the sole nitrogen source was isolated with pyruvate and sulfate as the energy sources by Preuss *et al.* (1993). TNT was biologically reduced to TAT; the pathway proceeded via DANT to diaminohydroxylaminotoluene and then to TAT. TAT was further degraded to unidentified products, presumably with release of nitrogen. Inhibition studies suggested that dissimilatory sulfite reductase might be responsible for reduction of DANT and/or diaminohydroxylaminotoluene. TAT was found to further degrade spontaneously under aerobic conditions in the presence of certain metal ions, especially manganese.

F. Fungal Transformation and Degradation of TNT

There is considerable interest in the fungal degradation of TNT; many microorganisms are able to reduce TNT, but liginolytic fungi are the only organisms isolated to date which can truly mineralize TNT. Mineralization is usually measured by monitoring the release of $^{14}CO_2$ during growth on uniformly ring-labeling ^{14}C-TNT. This does not necessarily mean that the organism is utilizing the TNT as a carbon and energy source. It is more likely that TNT degradation is via a co-metabolic process rather than via primary metabolism; therefore other carbohydrates are used as carbon sources for energy and biomass production. All investigations of TNT transformation by fungi that have been described to date suggest that the initial attack is by reduction of at least one nitro group of the explosive to either 4- or 2ADNT via the nitroso-DNT and HADNTs intermediates. Subsequently, the transformation of the products is dependent on several parameters such as fungal species, culture condition, and time. Numerous studies have demonstrated the effectiveness of fungi in transforming TNT. For example, Parrish (1977) screened 190 fungal strains from 98 genera and found that 183 of these were able to transform TNT to a significant degree when cultured in liquid medium supplemented with 100 mg/liter TNT. The major products identified were 4A26DNT, 4HA26DNT, and 2,2′-6,6′-tetranitro-4,4′-azoxytoluene. This suggested that the transformation of TNT proceeded via a similar route to that observed in bacteria (Fig. 3), and probably involved a nitroreductase-like activity. Use of [^{14}C]TNT gave no evidence for ring cleavage products. Interestingly, the ability to transform 2,4-dinitrotoluene (24DNT) was much less common, occurring to a significant degree in only 5 of the strains tested. Eight fungal strains belonging to various taxonomic groups were tested by Bayman and Radkar (1997). Again, the reduction of TNT to HADNT,

ADNT, and tetranitroazoxytoluenes was noted; a few strains produced water-soluble products, but none mineralized TNT to a significant degree. Mineralization was observed from a study by Scheibner *et al.* (1997). In this investigation, 91 fungal strains from 32 genera rapidly reduced TNT to ADNT, but only basidiomycetes, which are involved in wood and litter decay, showed significant mineralization of TNT (42% mineralization of 0.1 m*M* [^{14}C]TNT over 64 days). Meharg *et al.* (1997) also reported that ectomycorrhizal basidiomycetes and their extracellular enzymes were able to transform TNT.

White rot fungi species such as *Phanerochaete chrysosporium* and *Phlebia radiata* are able to degrade TNT. For example, Fernando *et al.* (1990) and Fernando and Aust (1991) reported that *P. chrysosporium* rapidly transformed high levels of [^{14}C]TNT in liquid cultures and in soil. In liquid culture, with a starting concentration of 100 mg/liter TNT, 14% of label was recovered as $^{14}CO_2$ and 52% as water-soluble products, 5% was associated with the mycelium, and 22% remained as TNT after 30 days. In soil with 10,000 mg TNT/kg soil, after 90 days, 18% of label was recovered as $^{14}CO_2$ and 15% as TNT, and 12% was bound to the matrix and could not be extracted. Partial mineralization of TNT by growing mycelia of *P. chrysosporium* was also reported by Spiker *et al.* (1992); interestingly, pregrown mycelia transformed TNT but did not mineralize it. Bumpus and Tatarko (1994) showed that *P. chrysosporium* initially reduced TNT to HADNT and ADNT, and these were further degraded with partial release of CO_2. Thus the mineralization of these reduced products is considerably more effective than the mineralization of TNT itself (Hess *et al.,* 1998).

III. Nitramine Explosives

A. Chemistry and Fate of Nitramines in the Environment

Nitramines have been shown to be susceptible to two chemical treatment processes; alkaline hydrolysis and reductive denitration. The alkaline hydrolysis of RDX is initiated by the removal of nitrite, resulting in an unstable product which spontaneously breaks down to give 1.6 *M* NO_2^-, 1.5 *M* $HCOO^-$, 0.1 *M* CH_3COO^-, 1.1 *M* HCHO, 0.9 *M* NH_3, 1.1 *M* N_2O, and 0.34 *M* N_2 per mol of RDX (Heilmann *et al.,* 1996). In the reductive denitration of HMX the reaction begins with radical initiation by photolysis or an agent such as dithionite followed by the replacement of nitro groups by hydrogen through the action of a mild reducing agent such as 1-benzyl-1,4-dihydronicotinamide (Chapman *et al.,* 1996). The cleavage products produced via the radical anion are N-methylpicramide (N-methyl-2,4,6-trinitroaniline) from HMX. The predicted product of HMX reductive denitration would be

octahydro-1,3,5,7-tetrazocine; however, this compound is unstable and dissociates to form ammonia and formaldehyde, which form the more stable hexamethylenetetramine adduct.

After TNT, RDX is probably the explosive of greatest environmental concern. RDX is released into the environment mainly as a result of the manufacturing process during which up to 12 mg/liter RDX is released in process wastewaters (Jackson *et al.,* 1978). HMX contamination from open burning and detonation varied from 10 mg/kg to 1.6 g/kg at one antitank firing range (Jenkins *et al.,* 1998; Thiboutot *et al.,* 1998). RDX and HMX have both been shown to be toxic to mammals, having an adverse effect on the central nervous system, and they are classified as class C carcinogens (Rocheleau *et al.,* 1999; Talmage *et al.,* 1999; Yinon, 1990). RDX has also been shown to be toxic to aquatic life, and an aquatic toxicity level of 0.3 mg/liter was set based on studies with algae, invertebrates, and fish (Sullivan *et al.,* 1979). Both RDX and HMX have been shown to persist in the environment with only limited degradation observed. No examples of heterocyclic nitramines have been found in nature; hence, RDX and HMX are considered to be true xenobiotic compounds. Under aerobic conditions, RDX is much less susceptible to biotransformation than TNT, is less strongly adsorbed to soil, and is therefore more mobile, giving rise to large plumes of contamination (Singh *et al.,* 1998). Under anaerobic conditions, RDX undergoes biological reduction, but unlike TNT it does not result in persistent reduced metabolites. In experiments comparing transformation of [^{14}C] RDX in aerobic and anaerobic soil slurry bioreactors, Shen *et al.* (1997) showed that under aerobic conditions RDX was recalcitrant, but under anaerobic conditions 15% of the RDX was mineralized to $^{14}CO_2$ by indigenous soil microorganisms. When the bioreactor was supplemented with anaerobic sewage sludge, there was an increase in RDX mineralization up to 60%.

In an anoxic system designed to remediate RDX and nitrate containing wastewater Freedman and Sutherland (1998) discovered that RDX was not transformed when nitrate was present. This indicated that RDX is not effectively reduced under nitrate-reducing conditions. This was also shown by Ronen *et al.* (1998), who reported the biodegradation of RDX and nitrate-containing munitions wastewater using a sequential anaerobic (denitrifying) and aerobic system. The complete removal of the RDX in the aerobic stage only occurred after complete removal of nitrate in the anoxic stage. In contrast to its usual recalcitrance, under aerobic conditions the RDX was apparently used as a nitrogen source, with cyclohexanone (a common component of RDX process wastewaters) being supplied as a carbon source.

Under sulfate-reducing conditions both RDX and HMX have been shown to be effectively degraded (Boopathy *et al.,* 1998c, 1998d). The

effective removal of RDX and HMX by composting has also been reported (Keehan and Sisk, 1996; Williams *et al.,* 1992).

B. Biodegradation of Nitramines under Anaerobic Conditions

The first significant insights into the anaerobic degradation of RDX were provided when McCormick *et al.* (1981) demonstrated that under anaerobic conditions in nutrient broth, mixed cultures from sewage sludge rapidly degraded RDX. This degradation was accompanied by the transient accumulation of the mono-, di-, and trinitroso derivatives, which were converted to products including formaldehyde, methanol, hydrazine, 1,1-dimethylhydrazine, and 1,2-dimethylhydrazine (Fig. 8).

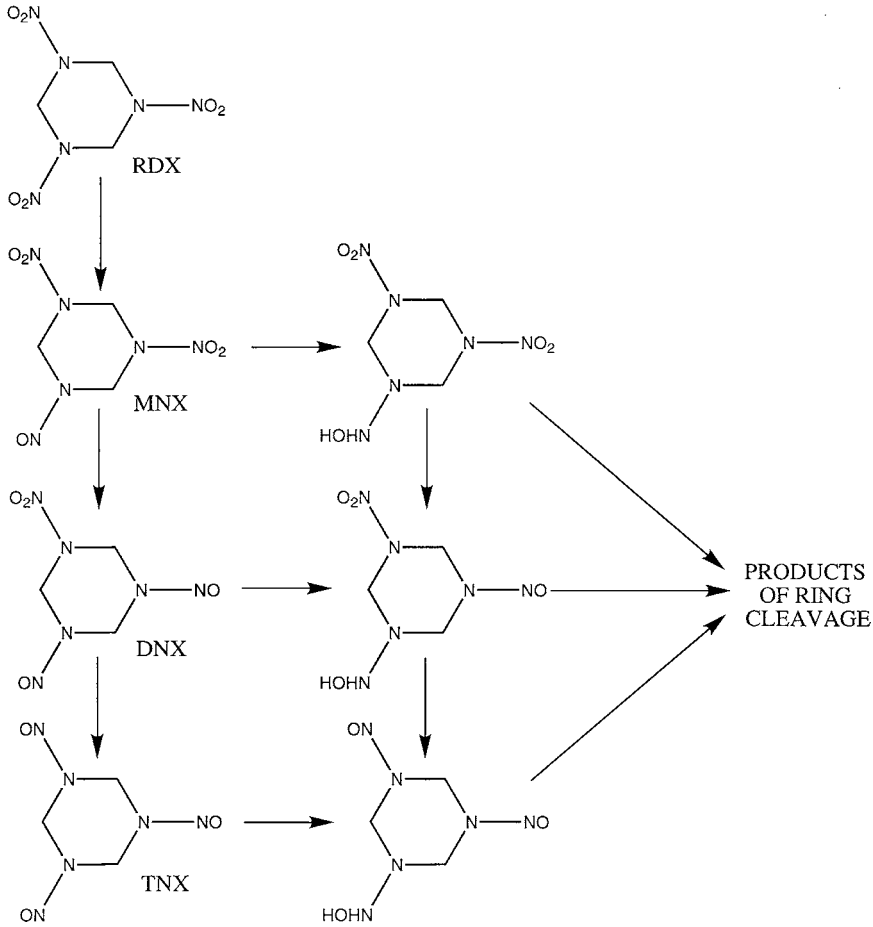


Fig. 8. Sequential reduction of RDX via nitroso derivatives and putative hydroxylamino derivatives. MNX: mononitroso-RDX; DNX: dinitroso-RDX; TNX: trinitroso-RDX.

No radioactivity from [^{14}C] RDX was recovered as $^{14}CO_2$, and it was proposed that the reduction of a nitroso group to a dihydroxylamino group resulted in products which were unstable and subsequently underwent hydrolytic cleavage and rearrangement. This pathway was reinforced by further studies undertaken by McCormick *et al.* in 1985 when they also observed the degradation of HMX by a similar mechanism, although in this instance only the mono- and dinitroso intermediates were observed. Young *et al.* (1997b) also reported a similar degradation process when indigenous soil microorganisms catalyzed the breakdown of RDX in a soil slurry reactor supplemented with corn steep liquor. The results of these studies show that RDX can be broken down; however, the nitrosamines and hydrazines are toxic, mutagenic, and carcinogenic, and are therefore not ideal products for bioremediation purposes.

Recently Hawari *et al.* (2000a) reported the degradation of RDX in liquid cultures containing municipal sludge by at least two routes of degradation. The first route involved the production of the nitroso derivative intermediates hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine and hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (Fig. 8). The second degradation pathway involved the production of the ring cleavage intermediates methylenedinitramine and bis(hydroxymethyl)nitramine (Fig. 9). Using ring-labeled ^{15}N-RDX Hawari *et al.* (2000a) demonstrated that the ring nitrogens were incorporated into these intermediates with two-ring nitrogens per methylenedinitramine, one-ring nitrogen per bis(hydroxymethyl)nitramine, and one-ring nitrogen per N_2O. None of the metabolites described were persistent and all disappeared, giving rise to nitrous oxide, formaldehyde, methanol, and formic acid, which in turn was broken down to methane and carbon dioxide. Hawari *et al.* (2001) also showed the degradation of HMX via the same biotransformation routes as its congener RDX. A proportion of the HMX was reduced to the nitroso derivatives octahydro-1-nitroso-3,5,7-trinitro-1,3,5,7-tetrazocine and octahydro-1,3-dinitroso-5,7-dinitro-1,3,5,7-tetrazocine (and its isomer octahydro-1,5-dinitroso-

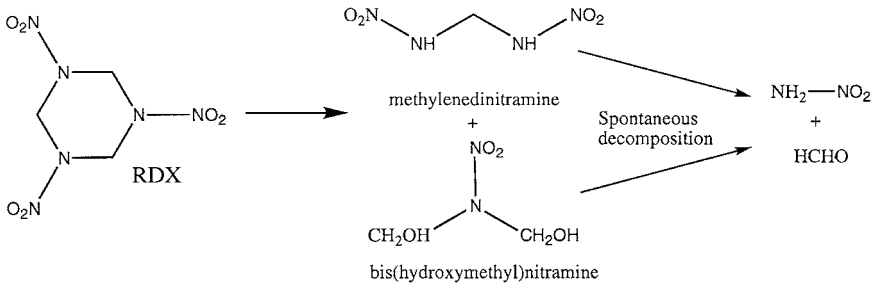


FIG. 9. Ring cleavage of RDX under anaerobic conditions.

3,7-dinitro-1,3,5,7-tetrazocine) (Fig. 10). The nitroso derivatives were further degraded, although the further products formed were not identified. In parallel with RDX, a proportion of the HMX was observed to form ring cleavage products which were tentatively identified as methylenedinitramine and bis(hydroxymethyl)nitramine (Fig. 11). These intermediates were further degraded to form nitrous oxide and formaldehyde.

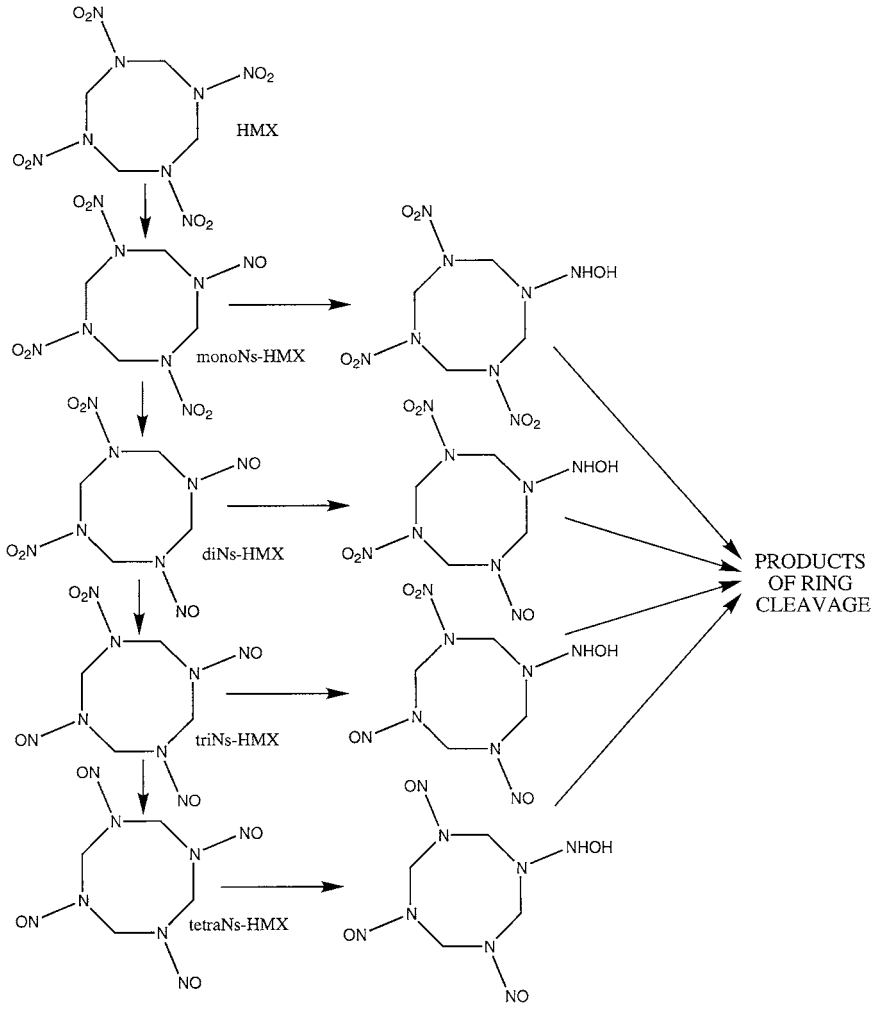


FIG. 10. Sequential reduction of HMX via nitroso derivatives and putative hydroxylamino derivatives.

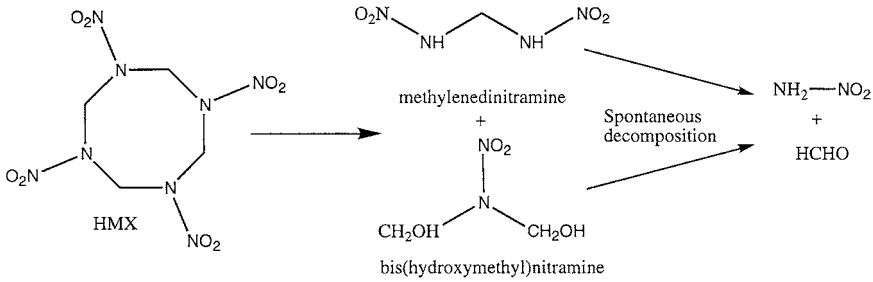


FIG. 11. Ring cleavage of HMX under anaerobic conditions.

Anaerobic degradation of RDX by pure bacterial cultures has been reported on a number of occasions and appears to occur in a manner similar to that reported by McCormick *et al.* (1981). Kitts *et al.* (1994) reported the isolation of organisms from RDX- and HMX-contaminated soil. Three organisms, identified as members of the Enterobacteriaceae, were shown to degrade RDX and HMX in pure culture. One isolate, *Morganella morganii,* could degrade HMX in the presence of RDX, whereas the other two isolates, *Providencia rettgeri* and *Citrobacter freundii,* could only degrade HMX in the absence of RDX. There were also differences in the extent of biotransformation observed. *Morganella morganii* and *P. rettgeri* showed complete degradation of RDX and its nitroso intermediates, while *C. freundii* only partially degraded RDX with the production of high concentrations of nitroso intermediates. HMX was also transformed to the nitroso derivatives, but at a lower rate, possibly reflecting its lower solubility. Bacteria were isolated from a RDX degrading consortium and investigated for their ability to transform RDX (Young *et al.,* 1997a). The isolates were identified as *Pseudomonas putida, Serratia marescens, Klebsiella pneuminiae, Xanthomonas maltophilia* (= *Pseudomonas maltophilia* = *Stenotrophomonas maltophilia*), and *Escherichia coli.* Following aerobic growth the isolates were transferred into an anaerobic phase, during which all transformed RDX, with *S. marcescens* proving the most efficient. Kitts *et al.* (2000) showed that the ability of the Enterobacteriacae family to transform RDX may be due to the action of the same oxygen-insensitive nitroreductases as are involved in TNT reduction (see Section IIB), although the activity of the isolated enzyme against RDX was very low.

Sulfate-reducing bacteria have also been shown to degrade RDX and HMX. Boopathy *et al.* (1998c, 1998d) demonstrated that a consortium of several *Desulfovibrio* spp. were able to utilize RDX and HMX as sole nitrogen sources. The nitrogen released from these compounds being converted to ammonia before assimilation.

C. Biodegradation of Nitramines under Aerobic Conditions

The majority of studies described to date have found RDX and HMX to be persistent under aerobic conditions, but recently there have been reports of their aerobic metabolism. The mineralization of RDX by a consortium under aerobic conditions was shown by Thiboutot *et al.* (1994). The aerobic mineralization of RDX was also reported by Ronen *et al.* (1998) in a wastewater treatment process on the addition of cyclohexanone as an additional carbon source. The formation of five nitroso derivatives (two di-isomers, and one of each of mono-, tri-, and tetranitroso derivatives) were observed during aerobic treatment of HMX in water using manure and indigenous organisms from contaminated land (Harkins, 1998). Recently pure cultures have been shown to utilize RDX as a sole nitrogen source under aerobic conditions. Binks *et al.* (1995) isolated a strain of *Stenotrophomonas maltophilia* (designated PB1) from explosive contaminated soil which apparently used three of the six available nitrogen atoms of RDX for growth. The degradation was inducible and inhibited by the presence of ammonium nitrate. Two metabolites were observed and identified as methylenedinitramine and methylenedinitramine chloride salt (Binks *et al.,* 1995; Hawari *et al.,* 2000a).

A *Rhodococcus* sp. strain DN22 isolated from RDX-contaminated soil was shown to degrade RDX aerobically (Coleman *et al.,* 1998). The isolate utilized RDX as sole nitrogen source using three out of the six available nitrogen atoms as previously reported for *S. maltophilia* PB1. The degradation of RDX was inhibited by the presence of ammonium, but not nitrite or nitrate, and the transformation would not occur under anaerobic conditions. In subsequent studies on RDX degradation by *Rhodococcus* sp. DN22 cytochrome P-450 activity was implicated (Coleman and Duxbury, 1999). Cytochrome P-450 involvement was also implied by a recent study by Tekoah and Abeliovich (1999) on another *Rhodococcus* sp. YH11. These studies did not result in the identification of any metabolites other than nitrite.

D. Biodegradation of Nitramines by Fungi

Fungal degradation of RDX has not been studied extensively, but Fernando and Aust (1991) showed that in liquid culture the white-rot fungus mineralized 67% of 0.028 mg/liter [^{14}C] RDX in 30 days with only 4% RDX recoverable. In soil the extent of mineralization was increased to 76% with the addition of corn cob mix. No intermediates were detected and no degradation pathway was suggested. The degradation of RDX by *P. chrysosporium* was also suggested by Sublette *et al.*

(1992) who investigated the remediation of "pink water" contaminated with 150 mg/liter TNT and 25 mg/liter RDX using a rotating biological contactor system. In a study by Bayman *et al.* (1995) the RDX degrading ability of *P. chrysosporium* was compared with three other fungal species. The three species examined were *Cyanthus pallidus,* another lignin-degrading basidiomycete; a zygomycete, *Cunninghamella echinulata* var *elegans*; and an ascomycete, *Cladosporium resinae*. The fungi were grown in a nutrient poor nonlignolytic medium containing 100 mg/liter RDX. All four fungi showed RDX disappearance and over the initial three days extractable RDX decreased by 22% with *P. chrysosporium* and the other fungi showing RDX decreases of between 12% (*Cu. echinulata*) and 31% (*Cl. resinae*). For all of the fungal species the radiolabel from the [^{14}C] was only recovered from the organic fractions rather than the aqueous or gaseous fractions, and there was no radioactivity associated with cell wall material. The results obtained were in contrast to those of Fernando and Aust (1991) as there was no mineralization observed. In a more recent study also using *P. chrysosporium,* Sheremata and Hawari (2000) showed that 52.9% of RDX was mineralized after 60 days with glycerol used as a carbon source. The only RDX intermediate detected was hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine with high yields of nitrous oxide and carbon dioxide obtained after a two-day lag period. It was noted that the appearance of the nitrous oxide and carbon dioxide correlated with the appearance of manganese peroxidase enzyme activity, which was suggested to be involved in the RDX degradation.

IV. Nitrate Ester Explosives

A. Chemistry and Fate of Nitrate Esters in the Environment

Nitrate esters are known to persist in the environment for significant time periods, probably as a result of their comparative stability. It is possible to hydrolyze nitrate esters under acidic or alkaline conditions, resulting in the formation of the aldehyde and nitrite, rather than (or in addition to) the alcohol and nitrate (Boschan *et al.,* 1955; Urbanski, 1965). This contrasts with the reactions of the sulfate and phosphate esters, which are easily hydrolyzed to the alcohol and sulfate or phosphate (White and Snape, 1993). The differences presumably reflect the strong electron withdrawing nature of the nitrate ester group. The nitrate esters are uncharged and therefore have relatively low aqueous solubility (Rosenblatt *et al.,* 1991), which may also have a role in their relative stability to hydrolysis. The bulk of evidence suggests that it is in fact a reductive transformation of nitrate esters which actually occurs

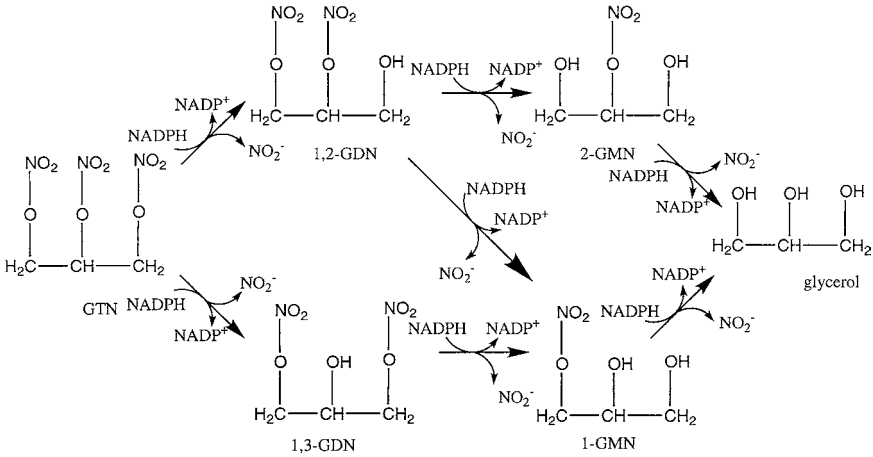
in the environment with the formation of alcohol and the liberation of nitrite.

Nitrate esters have been manufactured over the last hundred years for use primarily as explosives, but more recently medicinally as vasodilators. The sole example of a biologically derived nitrate ester appears to be an alkenyl nitrate that acts as an insect sex pheromone (Hall *et al.,* 1992). The chemical interaction of NO_x and hydrocarbons in the atmosphere can give rise to some organic nitrates (Roberts, 1990), but the multiple substituted nitrate esters found in the environment today are almost certainly the result of their introduction by humans. Although nitrate esters are used therapeutically at low doses, at higher levels nitrate esters and their metabolites are toxic. Mammalian toxicity data for GTN suggest that acute toxicity occurs at levels of 30–1300 mg/kg (Wendt *et al.,* 1978), and Urbanski (1984) reports an LD_{50} of 1 mg/liter for fish.

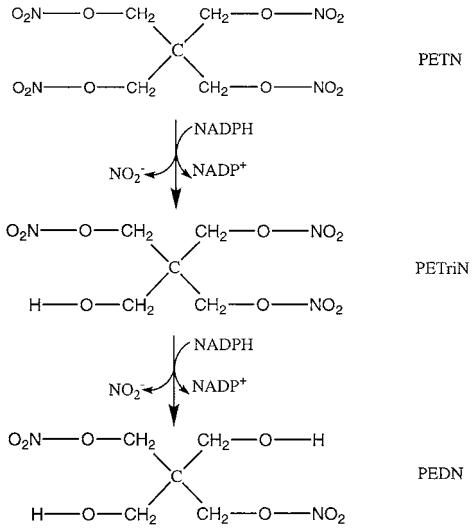
B. Bacterial Metabolism of Nitrate Esters

A number of microorganisms have been shown to degrade nitrate esters via sequential denitration, resulting in the production of the parent alcohol, and the release of nitrogen detected as nitrate, nitrite or nitric oxide. The metabolism of nitrate esters by bacteria has only been characterized relatively recently, and when White *et al.* published their review in 1993, the best described reports of bacterial degradation were of GTN by activated sludge (Wendt *et al.,* 1978) and ethylene glycol dinitrate (EGDN) by a strain of *Klebsiella oxytoca* (Tan-Walker, 1987). In both examples the nitrate groups were removed sequentially, but the degradation of the mononitrate was not detected.

Recently there have been a number of reports of mixed microbial cultures such as activated sludge sequentially denitrating GTN to produce glycerol-1,2-dinitrate (12GDN) and glycerol-1,3-dinitrate (13GDN), followed by glycerol-1-mononitrate (1GMN) and glycerol-2-mononitrate (2GMN) and eventually glycerol (Fig. 12A) (Christodoulatos *et al.,* 1997; Bhaumik *et al.,* 1997, 1998; Accashian *et al.,* 1998). Wastewater from a propellant manufacturing plant, containing GTN at a level of around 180 mg/L, was treated in a pilot scale batch reactor, the GTN being degraded to below detection levels. The microbial community utilized ethyl acetate present in the wastewater as a carbon source. The reactor cycle involved an 8-h aerobic phase followed by a 5-h anoxic phase. The GTN level fell throughout the aerobic phase and the resultant nitrate generated was removed during the anoxic phase (Pesari and Grasso, 1993). The authors suggested that GTN was also amenable to degradation during the anoxic phase, though the extent of denitration was not reported.



(A)



(B)

FIG. 12. Sequential denitration of nitrate esters. The enzymatic reduction of GTN (A) and PETN (B) are shown. PEDN: pentaerythritol dinitrate.

The complete mineralization of GTN under anaerobic conditions was demonstrated in sealed microcosms inoculated with anaerobic sludge from a municipal waste treatment plant (Christodoulatos *et al.,* 1997). The dinitrate and mononitrate intermediates were observed, but there was no apparent accumulation of nitrate or nitrite. The rate of GTN degradation was significantly enhanced when glucose was added as a carbon source. The complete degradation of GTN by both anaerobic and aerobic sludge was shown by work with batch and packed bed reactors (Bhaumik *et al.,* 1997). However, degradation under aerobic conditions was slow and required the substantial addition of a cosubstrate (Bhaumik *et al.,* 1998). In general, the sequential denitration steps proceeded at decreasing rates, with GMN frequently accumulating, and an additional carbon source usually required, possibly as a result of the slow removal of the final nitrate group. However, Accashian *et al.* (1998) reported that a mixed culture selectively enriched from the aeration tank sludge of a GTN treatment facility could utilize GTN as a sole carbon and nitrogen source.

The aerobic degradation of nitrate esters by pure cultures of bacteria has recently been investigated. Screening bacteria isolated from soil samples heavily contaminated with nitrate ester explosives resulted in the identification of a number of bacteria which could use GTN as a sole nitrogen source or were tolerant of high GTN concentrations. Two strains were identified as *Bacillus thuringiensis/cereus* and *Enterobacter agglomerans,* and demonstrated a correlation between loss of GTN with accumulation of nitrite in whole cell assays (Meng *et al.,* 1995). The metabolites were identified as 12GDN, 13GDN, 1GMN, 2GMN, and glycerol; all nitrogen released was accounted for as nitrite and/or nitrate. Subsequent work on the *Bacillus* sp. (ATCC 51912) showed that the strain was also able to sequentially denitrate propylene glycol dinitrate (PGDN) to propylene glycol mononitrate (PGMN) and eventually propylene glycol after prolonged incubation (Sun *et al.,* 1996). Selective enrichments using GTN as a sole nitrogen source and glycerol as an additional carbon source resulted in the isolation of a *Pseudomonas* sp. from a river sediment (White *et al.,* 1996a) and an *Agrobacterium radiobacter* strain from activated sludge (White *et al.,* 1996b). The soil surrounding an explosives factory yielded a strain of *Arthrobacter ilicis* capable of denitrating EGDN to ethylene glycol mononitrate (EGMN) and ethylene glycol (Ramos *et al.,* 1996). The strain was isolated in coculture with a strain of *Agrobacterium radiobacter* capable of oxidizing ethylene glycol, thus enhancing mineralization. Enrichment cultures from explosive contaminated soil resulted in the isolation of a strain of *Enterobacter cloacae,* designated PB2, capable of growth on PETN or GTN as sole nitrogen source (Binks *et al.,* 1996). Recently strains of

P. putida and *P. fluorescens* capable of utilizing GTN as sole nitrogen source were isolated from GTN-contaminated soil (Blehert *et al.,* 1997; Blehert *et al.,* 1999).

C. Bacterial Nitrate Ester Reductases

Further characterization of the bacteria demonstrating nitrate ester degradation activity has led to the identification of a group of very similar enzymes. The enzymes catalyze the nicotinamide cofactor dependent reductive cleavage of nitrate esters to give an alcohol and nitrite (Fig. 12). For example, the enzyme responsible for the denitration of PETN and GTN in *Enterobacter cloacae* PB2, designated PETN reductase, was found to be a soluble monomeric Flavin mononucleotide (FMN) binding flavoprotein of 40 kDa which required NADPH for activity. The gene encoding this enzyme, designated *onr* (organic nitrate reductase) was cloned and sequenced (French *et al.,* 1996). The sequence revealed that PETN reductase was related to the Old Yellow Enzyme family of flavoenzymes. In a more recent study French *et al.* (1998) showed that PETN reductase could also reduce TNT to its hydride–Meisenheimer complex and further to the dihydride adduct (Sec. II.B). Due to its interesting activities and its potential for bioremediation applications, PETN reductase has undergone considerable further investigation and a crystal structure has been determined (Moody *et al.,* 1998). Transgenic tobacco plants expressing PETN reductase were prepared and were shown to tolerate concentrations of GTN and TNT which were inhibitory to the germination of wild-type seedlings (French *et al.,* 1999). The transgenic plants also showed greatly enhanced denitration of GTN to GDN and GMN demonstrating the potential of combining the impressive biodegradative capabilities of soil bacteria with the stability and high biomass of plants for *in situ* bioremediation.

Similarly, the enzyme responsible for GTN degradation in the strain of *Agrobacterium radiobacter* isolated by White *et al.* (1996a) was purified and the structural gene cloned and sequenced (Snape *et al.,* 1997). The enzyme, GTN reductase, was very similar to PETN reductase in its sequence and properties. It did, however, differ in that it required NADH as the source of reducing equivalents and was induced by the presence of GTN, whereas PETN reductase utilizes NADPH and is expressed constitutively. Enzymes similar to PETN reductase and GTN reductase have also been characterized from two *Pseudomonas* strains able to utilize GTN as a sole nitrogen source for growth (Blehert *et al.,* 1997). The cloning and sequencing of the genes encoding these enzymes, designated *xenA* and *xenB,* revealed their close relationship to PETN reductase, GTN reductase, and other Old Yellow Enzyme homologues

(Blehert *et al.,* 1999). Thus, these enzymes constitute a novel family of nitrate ester reductases. Far from having evolved specifically to deal with the presence of explosives in the environment, it is now apparent that the enzymes of this family are widespread in bacteria. An *E. coli* homologue, NemA, has been purified as an N-ethylmaleimide reductase (Miura *et al.,* 1997). The closest relative of PETN reductase is morphinone reductase, which converts the alkaloid morphinone to hydromorphone (French and Bruce, 1994). It has been suggested that these enzymes may have a role in the detoxification of pro-oxidant substances (Blehert *et al.,* 1999). This has also been suggested for the bacterial oxygen-insensitive nitroreductases which are also small FMN-binding flavoproteins with a broad substrate specificity. Given how widespread these enzymes are, the variability of bacterial tolerance and growth on nitrate esters might be the result, at least in part, of differences in nitrite/nitrate metabolism or toxicity of metabolites rather than the initial enzyme activity.

The denitration of GTN can result in the production of two isomers of the di- and mononitrate, which has been suggested as evidence of multiple degradation pathways (Bennett *et al.,* 1994) or multiple enzymes with varying regiospecificities (White and Snape, 1993). However, it is clear that the regiospecificity of GTN metabolism exhibited by bacterial nitrate ester reductases varies from species to species, despite significant similarity in enzyme structure. When *A. radiobacter* was grown on GTN as a sole nitrogen source, the denitration of the C2 nitrate group by resting cells was approximately ten times faster than denitration of the terminal nitrate groups (White *et al.,* 1996). The ratio of GDN products was the same when the purified nitrate ester reductase from the same organism was used (Snape *et al.,* 1997). The purified enzyme from *P. fluorescens* produced a similar bias towards the 2 position of GTN and 1,2-GDN (Blehert *et al.,* 1999). However, the denitration of GTN by the purified *P. putida* nitrate ester reductase resulted in the 2:1 ratio of 1,2- to 1,3-GDN expected if there were no regioselective bias. The differences in regiospecificity therefore appear to be the result of very subtle differences in enzyme substrate recognition.

Work on nitrate ester degradation by *Bacillus* sp. ATCC 51912 and an *E. agglomerans* sp. suggested that the nitrate ester reductases may not be the only enzymes involved in the bacterial transformation of nitrate esters (Meng *et al.,* 1995). Expression of the enzymes involved was constitutive and cytosolic in the *Bacillus* sp., but membrane associated in the *E. agglomerans* sp. Enzyme assays carried out on both cell extracts, postdialysis, suggested that diffusible cofactors such as NADPH were not required for activity. This is surprising since it seems to suggest a hydrolytic mechanism resulting in the release of nitrate instead

of a reductive denitration releasing nitrite. The authors suggested that nitrogen was initially released as nitrate and then reduced to nitrite by a nitrate reductase. The complete degradation of GTN required the repeated addition of cell extract, possibly suggesting the involvement of high molecular weight redox proteins. Such reducing equivalents would be retained by dialysis but would be rapidly depleted *in vitro* without a regeneration system.

D. Fungal Metabolism of Nitrate Esters

The first reported fungal degradation of GTN was by the filamentous fungus *Geotrichum candidum* (DuCrocq *et al.,* 1989). High performance liquid chromatography (HPLC) analysis showed the production of large quantities of dinitrates followed by the mononitrates, suggesting the sequential removal of nitrate groups as observed in bacteria. The ability of the white rot fungus *Phanerochaete chrysosporium* to degrade GTN was investigated by DuCrocq *et al.* (1990) and again GTN was transformed to GDN and GMN. Subsequent more detailed analysis revealed that at least two enzyme activities were responsible for GTN degradation in *P. chrysosporium* (Servent *et al.,* 1991, 1992). The first activity was an oxygen-insensitive glutathione-dependent enzyme located in the cytosol-liberating nitrite, which was subsequently converted into nitrate. The second enzyme was an oxygen-sensitive NADPH-dependent cytochrome P450-like activity catalyzing the liberation of nitric oxide. There was no evidence observed for a hydrolytic mechanism resulting in nitrate formation. *P. chrysosporium* has been tested for bioremediation purposes immobilized in a packed bed reactor (Bhaumik *et al.,* 1997). The system achieved 99% removal of the GTN, leaving a mixture of dinitrate and mononitrate esters. In the presence of supplementary carbon and nitrogen sources, the fungus *Penicillium corylophilum* was able to denitrate GTN with the complete disappearance of GDN and GMN (Zhang *et al.,* 1997).

The nitrate ester nitrocellulose is very insoluble in aqueous solutions and therefore biodegradation is limited by its poor bioavailability. There are, however, several reports of fungal degradation of nitrocellulose. *Aspergillus fumigatus* grew in minimal media containing glucose as a carbon source and a suspension of nitrocellulose as the sole nitrogen source (Brodman and Devine, 1981). A co-culture of *Sclerotium rolfsii* and *Fusarium solani* was shown to degrade nitrocellulose measured as a decrease in acetone-extractable material in the culture medium (Sharma *et al.,* 1995a). The fungus *Penicillium corylophilum* Dierckx isolated from a propellant containing nitrocellulose and GTN was able to partially degrade nitrocellulose when starch or xylose was added as

an additional carbon source (Sharma *et al.,* 1995b). In each case the biochemical basis for the activities has not been determined.

V. Conclusions

The discovery of widespread explosives contamination of soil, sediment, and groundwater has led to increasing concern about the persistence of these toxic compounds in the environment. Work published in recent years has shown that microorganisms have the metabolic capability to transform and degrade explosives, which were previously thought to be recalcitrant to biological attack. Much effort is now being directed at the characterization of the pathways responsible on a biochemical and genetic level. The selective pressure of environmental pollution is clearly developing microorganisms that might be harnessed for explosives removal by biotechnological processes. Nevertheless, the fact that explosives persist in the environment emphasizes the problems of the existing catabolic activities in dealing with this problem. Screening natural diversity is unlikely to yield organisms that satisfy all the demands for the biodegradation of explosives, since these compounds have only been present in the environment for tens of years and microorganisms have, therefore, had little time to evolve enzymes suited to this task. The application of genetic engineering and biochemical techniques promises to improve and evolve natural biodegradative capabilities further. Metabolic engineering may also have a considerable influence on the development of bioremediation systems in the near future; while this field is still a very young one, much has been accomplished and this is beginning to have an impact on environmental biotechnology. Recently, attention has focused on the use of transgenic plants for phytoremediation. Identification of the genes encoding explosive degrading enzymes now offers the exciting possibility of engineering plants to rapidly degrade these toxic pollutants.

Acknowledgments

The work in the authors' laboratories has been sponsored by DERA, BBSRC, and the MOD.

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Biodiversity of Acidophilic Prokaryotes

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I. Introduction

Interest in the biodiversity of "extreme" environments has grown over the past several years for several reasons, including the theory that such conditions were predominant on the young planet earth. Thus, early life forms may have consisted of organisms adapted to such environments, the so-called extremophiles. The same line of reasoning underlies the search for life forms from outside our planet. Other reasons for exploring the biodiversity of extreme environments are of a more applied nature, including the use of thermostable enzymes in industrial applications (Huber and Stetter, 1998).

One group of extremophiles that is becoming increasingly important, both ecologically and economically, is acidophilic microorganisms. Acidophiles can be found in all three domains of organisms (Fig. 1), indicating that the ability to thrive in acid environments developed early in evolution. These organisms thrive in environments of low pH (<3), and indeed are often the cause of the acidity in the environments in which they live. For example, mining activity exposes sulfidic minerals to microbially mediated attack leading to the solubilization of the sulfides.

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 49

0065-2164/01 $35.00

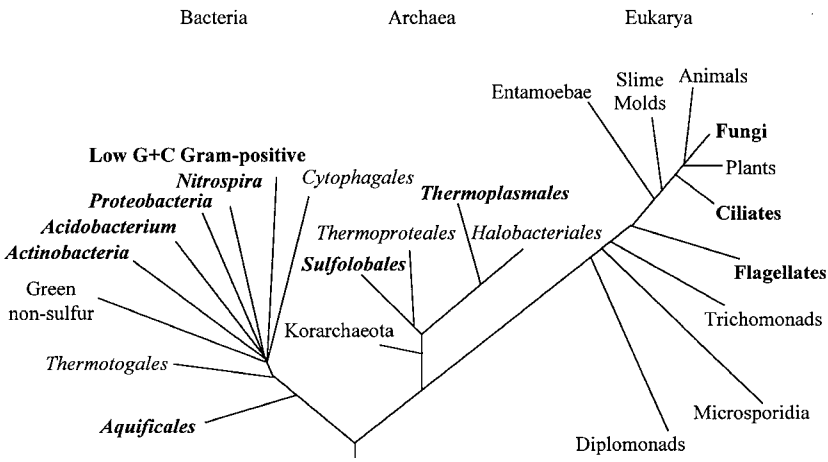

FIG. 1. A phylogenetic tree showing all three kingdoms of living organisms. Those that contain acidophilic organisms are highlighted in bold. The tree is derived from 16S (bacteria and archaea) and 18S (eukarya) rRNA sequences.

The ultimate end products of this attack are heavy metal laden acidic water that is often referred to acid mine (or rock) drainage (AMD/ARD). These acidic waters often flow into unpolluted streams and rivers and coat the bottom of rivers or lakes with ferric iron, and also bring with them metals that are toxic to aquatic life.

This same process, however, has proved to be commercially viable for the recovery of metals, commonly called "biomining." During the microbially catalyzed oxidation of low-grade metal sulfides, metals with commercial value are solubilized and can easily be recovered. Acidophiles are also used to oxidize the metal sulfide surrounding precious metals, such as gold, to enhance recovery of the metal. The recovery of gold from some refractory ores, those which are difficult to treat by conventional metallurgical techniques, can be as high as 100% compared to that from the untreated ores.

Here we review recent literature concerning the biology of acidophilic prokaryotes (bacteria and archaea) specifically relating to acidophilic taxonomy and phylogeny. The use of 16S rRNA phylogeny has led recently to clarification in the taxonomic relationships of some acidophiles. Also, the continuing advances in the physiology of acidophiles is reviewed. Other aspects of acidophilic microbiology have been recently reviewed and include biomining (Rawlings, 1997), role of acidophiles in biogeochemical cycling of metals (Johnson, 1998b), and the biology of acidophilic eukaryotes (Deneke, 2000; Gross and Robbins, 2000; Gross, 2000).

II. Biodiversity of Acidophilic Prokaryotes

Acidophilic prokaryotic microorganisms are widely distributed within the domains Bacteria and Archaea. This section describes the physiological biodiversity of currently known species of acidophilic prokaryotes. As the number of recognized genera of acidophiles has increased, both due to improvement in the techniques used to isolate them and also due to changes in nomenclature brought about by phylogenetic analysis, we have decided to list the acidophiles covered in this review and assign them generic abbreviations that we suggest could be adopted by other researchers (Table I).

A. Bacteria

1. *Genus* Leptospirillum

Leptospirillum spp. form a distinct lineage within the deep-rooted *Nitrospira* division. All other bacteria currently included in this group are neutrophilic.

At present, the genus *Leptospirillum* includes two recognized species (Hippe, 2000), *L. ferrooxidans* (the type species is *L. ferrooxidans* $L15^T$ = DSM 2705^T, DSM = Deutsche Sammlung von Mikrooganismen und Zellkulturen GmbH), and *L. thermoferrooxidans*. The original strain was isolated from a copper mine in Armenia by Markosyan (1972), but since then *Leptospirillum*-like bacteria have been isolated from mine sites, bioleaching plants, and acid mine drainage from many parts of the world. All of the isolates have, in common, the ability to couple the oxidation of ferrous iron to the reduction of molecular oxygen. Differences between the redox couples of Fe^{2+}/Fe^{3+} (+770 mV at pH 2) and O_2/H_2O (+820 mV) are marginal, and unique among acidophiles and possibly all other bacteria, *Leptospirillum* spp. appear to use no other substances either as electron donor or acceptor. However, due to its relatively high substrate (Fe^{2+}) affinity, tolerance of ferric iron and moderately thermal (>40°C) environments, it is frequently the dominant iron-oxidizing organism in mineral-leaching environments. *Leptospirillum* spp. are particularly adept at accelerating the oxidative dissolution of pyrite (FeS_2), which is the most abundant of all sulfide minerals in the lithosphere.

Bacteria classified as "*L. ferrooxidans*" have been known for some time to fall into two groups (Harrison and Norris, 1985), based on G+C contents of chromosomal DNA (either 51–52 mol%, for the group which includes the original isolate, or 55–56 mol% for a second group), DNA homologies, and more recently from 16S rRNA gene sequencing.

Reclassification and designation of the higher G+C group as a novel genus is currently under proposal (D. E. Rawlings, University of

TABLE I

PROPOSED GENERIC ABBREVIATIONS FOR ACIDOPHILES AND THE RESPECTIVE PHYLOGENETIC DIVISION THEY ARE AFFILIATED WITH

Genus name	Proposed abbreviation	Phylogenetic division
Acidianus	Ad.	Sulfolobales
Acidilobus	Al.	Sulfolobales
Acidimicrobium	Am.	Actinobacteria
Acidiphilium	A.	α-Proteobacteria
Acidisphaera	As.	α-Proteobacteria
Acidithiobacillus	At.	β/γ-Proteobacteria
Acidobacterium	Ab.	Acidobacterium
Acidocella	Ac.	α-Proteobacteria
Acidomonas	Amn.	α-Proteobacteria
Alicyclobacillus	Alb.	Low G+C Gram positive
"Ferrimicrobium"	Fm.	Actinobacteria
Ferroplasma	Fp.	Thermoplasmales
Hydrogenobacter	H.	Aquifacales
Leptospirillum	L.	Nitrospira
Metallosphaera	M.	Sulfolobales
Picrophilus	P.	Thermoplasmales
Stygiolobus	Sg.	Sulfolobales
Sulfobacillus	Sb.	Low G+C Gram positive
Sulfolobus	S.	Sulfolobales
Sulfurisphaera	Ss.	Sulfolobales
Sulfurococcus	Sc.	Sulfolobales
Thermoplasma	Tp.	Thermoplasmales
Thiomonas	Tm.	β-Proteobacteria

Stellenbosch, personal communication). A putative third group/species has been identified in clone libraries obtained from an abandoned pyrite mine at Iron Mountain, California, though no representative isolate has yet been obtained (Bond *et al.,* 2000b).

A thermotolerant *Leptospirillum*-like bacterium was isolated from Kunashir island (located to the north of Japan) by Golovacheva *et al.* (1992). This bacterium shared morphological (highly motile curved rods) and physiological (strictly aerobic, obligately chemolithotrophic and autotrophic) characteristics with mesophilic *L. ferrooxidans*. The isolate, which had a chromosomal G+C content of 56 mol%, had low (27%) homology with the mesophilic type strain (the original Armenian strain), and would appear to be more closely related to the higher G+C leptospirilli. The name *L. thermoferrooxidans* was revived for this strain and has been formally recognized (Hippe, 2000), with the type strain given as L-88^T, in the culture collection at the Institute of Microbiology, Moscow, Russia.

Other thermotolerant *Leptospirillum*-like isolates have since been isolated, mostly from 40–50°C bioleaching operations. Again these appear

to fall into the higher G+C subgroup, though not all isolates with 55–56 mol% G+C are thermotolerant. One *Leptospirillum*-like isolate which grew at up to 50°C has been found to have a culture doubling time of about 2 h when grown on ferrous sulfate, which compares to 10–12 h for most mesophilic isolates. This strain was found to be the dominant iron-oxidizing bacterium in mixed cultures leaching pyrite at 45°C (Okibe and Johnson, 2001).

2. Hydrogenobacter acidophilus

Hydrogenobacter acidophilus belongs to an early branching division of eubacteria (Fig. 2). One of the closest phylogenetic neighbors to this acidophile is the neutrophilic *Aquifex pyrophilus*. This thermoacidophilic bacterium (temperature optimum and maximum 65 and ~70°C, respectively; pH optimum 3–4, and lower limit 2.0) grows

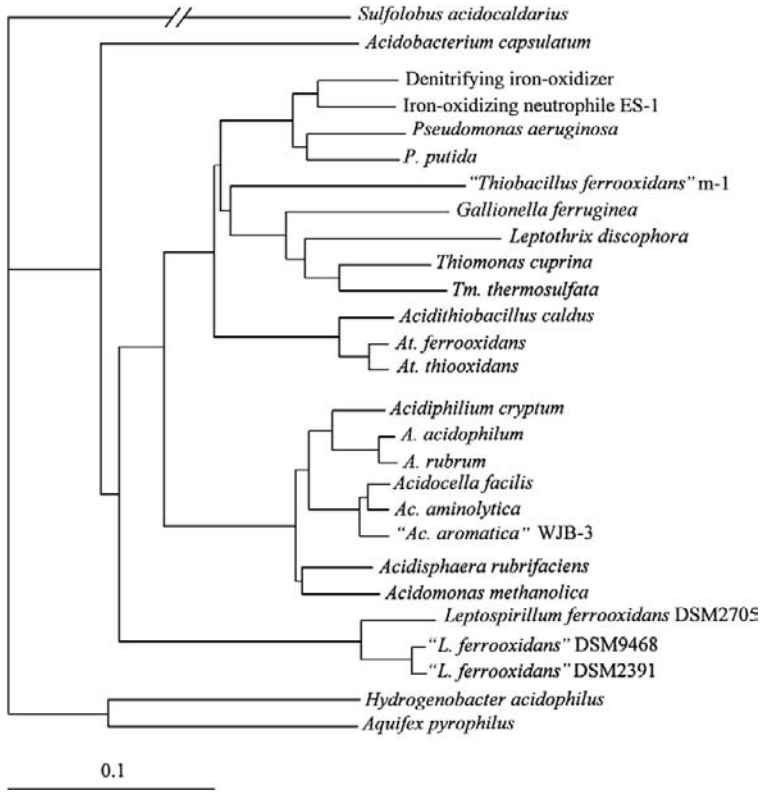

FIG. 2. Bacterial phylogenetic tree, based on 16S rRNA gene sequence, showing the relationship of Gram-negative acidophiles (in bold) to other Gram-negative bacteria. This tree was rooted with the 16S rRNA gene sequence from *S. acidocaldarius* as outgroup.

aerobically using hydrogen or elemental sulfur as electron donor (Shima and Suzuki, 1993). Originally isolated from a solfatara in Japan, this bacterium has also been isolated from sites in Yellowstone National Park, Wyoming (P. R. Norris, University of Warwick, UK, personal communication). This organism is the most thermophilic of all known acidophilic bacteria, though it is less acidophilic than sulfur-oxidizing *Acidithiobacillus* spp., including the moderate thermophile *At. caldus* (Sec. 4a) with which it would be predicted to compete in sulfur-containing niches (40–50°C; pH 2–3) where both can grow. There are no reports of *H. acidophilus* occurring in high temperature mineral oxidation plants where conditions (notably pH) may be too harsh for its survival. *Hydrogenobacter acidophilus* grows relatively rapidly (for an acidophilic autotroph), with a μ_{max} of 0.6 h^{-1} (corresponding to a culture doubling time of about 1.15 h).

3. Acidobacterium capsulatum

Another phylogenetically distinct acidophile is *Acidobacterium capsulatum*, which was the first microorganism of its phylogenetic division to be cultured (Hiraishi *et al.,* 1995). This is reflected in the fact that this bacterial division is now called the *Acidobacterium* division. Even though few bacteria from this division have been cultivated, 16S rRNA sequences ("environmental clones") that belong to this division have been obtained from many different environmental samples, including peat bogs and soil samples the world over.

Acidobacterium capsulatum was originally isolated from acid mine drainage (AMD) in Japan (Kishimoto *et al.,* 1991). The motile rods were noted to form a distinct capsule, and the isolate could be differentiated from other acidophilic heterotrophs by its production of menaquinone instead of ubiquinone in the electron transport chain. This bacterium is less acidophilic than other heterotrophic acidophiles listed below, with a pH range of 3–6 for growth. *Acidobacterium capsulatum,* like most *Acidiphilium* spp., forms colored (orange) colonies on solid medium, but this is not due to the presence bacteriochlorophyll, which has not been detected in this acidophile (Kishimoto *et al.,* 1995a).

Recently, bacteria sharing 94% 16S rDNA sequence homology with the original Japanese *Ab. capsulatum* isolate have been isolated from a "wetland" constructed at the site of the former Wheal Jane tin mine, Cornwall, UK (Hallberg and Johnson, 2001). One of these isolates, which appears to be the dominant heterotrophic acidophile in many samples taken from the man-made reed beds, forms salmon-pink colonies and, as the original isolate, grows better at pH 4 than at pH 3.

4. *Proteobacteria*

The *Proteobacteria* (formerly known as the purple bacteria) are by far the most numerous microorganisms that are known, either as isolates or as environmental clones. Acidophilic bacteria are found in three subgroups of this division.

a. α-Proteobacteria. Acidophiles within this subcategory are predominantly heterotrophic. Some may effect mineral dissolution either via an oxidative route (by modifying the activities of iron and sulfur oxidizers) or directly via reductive dissolution.

i. Genus *Acidiphilium.* *Acidiphilium* spp. appear to be the most widely distributed of all mesophilic, obligately heterotrophic bacteria found in metal-rich, acidic environments. Frequently, they occur as cryptic satellite organisms in cultures of iron- and sulfur-oxidizing chemolithotrophs. Most grow readily on acidified solid media, forming pigmented (cream, light brown, pink, and red) colonies.

Phylogenetic analysis based on 16S rDNA sequences segregates currently recognized *Acidiphilium* spp. into two distinct groups. The first includes the species first described by Harrison (1982), *A. cryptum,* as well as *A. organovorum* and *A. multivorum,* and the second group includes *A. acidophilum* (formerly *Thiobacillus acidophilus*), *A. rubrum,* and *A. angustum*. The validity of maintaining some of these bacteria as distinct species is questionable. For example, *A. cryptum, A. organovorum,* and *A. multivorum* share 99% homology of their 16S rRNA genes. Initially, physiological (especially nutritional) differences were used to differentiate these three acidophiles, but at least some of these have been found to be invalid in later experiments. For example, *A. cryptum,* which was thought to grow only in lean organic media, can grow to high cell densities ($<10^9$/ml, as does *A. organovorum* and *A. multivorum*) in organic-rich media which is supplemented with small amounts (typically 0.02% w/v) of yeast extract. There is even less justification for maintaining both *A. rubrum* and *A. angustum* as distinct species since these bacteria have >99% 16S rDNA sequence homology and share 100% DNA:DNA homology (Kishimoto *et al.,* 1995b).

One of the characteristics of all *Acidiphilium* spp. is their ability to reduce ferric iron to ferrous (Johnson and McGinness, 1991, and unpublished data). This may be observed using solid media containing ferric iron incubated aerobically with some organisms, such as *Acidiphilium* SJH (another member of the *A. cryptum* and *A. organovorum* group), though with others (e.g., *A. acidophilum* and *A. rubrum*) microaerobic conditions are required for ferric iron reduction to be evident. Most isolates can grow using ferric iron as terminal electron acceptor under

strictly anaerobic conditions, though both growth and ferric iron reduction tend to be more rapid when oxygen is present in small (<0.02 atm) concentrations. Kusel *et al.* (1999) isolated an acidophilic heterotroph from an acidic lake in eastern Germany by enrichment under strictly anoxic conditions. The bacterium, which coupled the oxidation of a wide range of electron donors (including hydrogen) to the reduction of ferric iron, was shown by 16S rRNA gene sequence analysis to be a strain of *A. cryptum*. Reductive dissolution of several ferric iron-containing minerals (e.g., magnetite, jarosite, and goethite) has been demonstrated with *Acidiphilium* SJH (Bridge and Johnson, 2000).

Kishimoto *et al.* (1995a) reported that four *Acidiphilium* spp. (*A. cryptum, A. organovorum, A. rubrum,* and *A. angustum*) produced bacteriochlorophyll-*a*. It was suggested that these acidophiles should be recognized as quasi-photosynthetic bacteria, although none of them grew using light as sole energy source. Later work revealed that the bacteriochlorophyll in *A. rubrum* contained a zinc porphyrin (Wakao *et al.,* 1996). Additionally, the presence of bacteriochlorophyll in bacteria formerly known as *Thiobacillus acidophilus,* which was known for some time to be more related to the *Acidiphilium* species (Lane *et al.,* 1992), has led to the reclassification of these bacteria as *Acidiphilium acidophilum* (Hiraishi *et al.,* 1998).

Among currently recognized *Acidiphilium* spp., *A. acidophilum* is unique in two respects: (1) its ability to fix inorganic carbon, as well as to assimilate organic carbon, and (2) by its propensity for oxidation of reduced inorganic sulfur compounds (RISCs). Oxidation of elemental sulfur and tetrathionate has also been observed, though to a more limited extent, with other *Acidiphilium* spp. (Hallberg *et al.,* 2001). *Acidiphilium acidophilum* displays considerably greater metabolic flexibility than other *Acidiphilium* spp., being able to grow heterotrophically on a wide range of organic substrates, mixotrophically using both inorganic and organic carbon, and autotrophically on various RISCs. This was the first *Acidiphilium* sp. to be isolated (Guay and Silver, 1975), though at the time it was classified (due to its growth with sulfur) as *Thiobacillus acidophilus*. As with many other *Acidiphilium* spp., its source was a supposedly pure culture of *Acidithiobacillus ferrooxidans*. However, unlike other *Acidiphilium* spp., which have subsequently been identified as environmental isolates in mine wastewaters (Johnson *et al.,* unpublished data), the original strain remains the only known representative of *A. acidophilum*.

ii. Genus *Acidocella*. A reassessment of the phylogenetic relationships of *Acidiphilium* spp. resulted in the transfer of two species ("*A. facilis*" and "*A. aminolytica*") to a new genus, *Acidocella* (Kishimoto *et al.,* 1995b). *Acidocella* spp. tend to be less acidophilic than *Acidiphilium*

spp. (lowest pH for growth typically ~2.5, rather than pH 2 or less for *Acidiphilium* spp.) and also less tolerant of some metals. *Acidocella* strain GS19h, however, was able to grow in the presence of 1*M* cadmium or zinc (Mahapatra and Banerjee, 1996). *Acidocella* spp. tend to be faster growing than *Acidiphilium* spp. and appear to be strict aerobes, though demonstration of anaerobic growth using ferric iron as electron donor is restricted by the sensitivity of some *Acidocella* spp. to ferric iron. Production of bacteriochlorophyll has not been observed with *Acidocella* spp., which tend to grow as bright white colonies on sold media.

Recent *Acidocella*-like isolates (coded strains WJB-3 and LGS-3) possess a novel and (so far) unique phenotype among heterotrophic acidophiles in that they are able to catabolize a variety of aromatic compounds, including benzoic acid, phenol, and naphthalene (Hallberg *et al.,* 1999). Somewhat curiously, this bacterium (proposed name "*Ac. aromatica*") appears unable to utilize organic substrates that are used by all other acidophilic heterotrophs (such as glucose and glycerol) though it can use fructose as the sole carbon/energy source, as well as a variety of aliphatic acids (Gemmell and Knowles, 2000).

iii. *Acidomonas methanolica.* *Acidomonas methanolica* is an acidophilic (range: pH 2.0–5.5) methylotroph (Urakami *et al.,* 1989). It shares several physiological characteristics in common with *Acidiphilium* spp., but is distinct phylogenetically from all other acidophilic genera. Methylotrophy itself is not uncommon amongst acidophilic heterotrophs: *A. multivorum* was found to grow on methanol (Wakao *et al.,* 1994), and we have noted (unpublished data) that all *Acidiphilium* spp. (but no *Acidocella* spp.) can be successfully subcultured in media containing methanol as sole (or major source, for those species that require yeast extract) carbon source.

iv. *Acidisphaera rubrifaciens.* *Acidisphaera rubrifaciens* is a relatively recent addition to the list of classified acidophilic heterotrophs (Hiraishi *et al.,* 2000). Isolates were obtained from hot springs and AMD in Japan, and grew as obligate aerobic cocci or coccobacilli (rather than rods, which is characteristic of other heterotrophic acidophilic genera) over a pH range 3.5–6.0. Light stimulates the growth of *As. rubrifaciens* and small amounts of zinc-containing bacteriochlorophyll are present. Isolates have been isolated from AMD in a former copper mine in Roeros, Norway, but they are only remotely related (94% identity of the 16S rRNA gene) to *As. rubrifaciens* (Johnson *et al.,* unpublished data).

b. β-Proteobacteria. *Thiomonas cuprina* (formerly "*Thiobacillus cuprinus*") was isolated from solfatara fields in Iceland and a uranium mine in Germany (Huber and Stetter, 1990). Isolates were found to

preferentially leach copper from some chalcopyrite ores, though this was not mediated via ferric iron as *Tm. cuprina* does not oxidize ferrous iron. This acidophile can grow heterotrophically on various organic compounds or autotrophically on elemental sulfur and RISCs, in common with *A. acidophilum,* though the two bacteria are phylogenetically distant from each other.

None of the other species of *Thiomonas* (*Tm. intermedia, Tm. perometabolis,* and *Tm. thermosulfata*) are acidophilic, though *Tm. thermosulfata* can grow at pH 4.3 (Shooner *et al.,* 1996). Recently, novel *Thiomonas*-like isolates have been isolated from ferruginous water draining a coal mine are in south Wales (Dennison *et al.,* 2001). These have been provisionally classified as "moderate acidophiles" as they grow at around pH 3 but not at pH 2. Like other *Thiomonas* spp. these isolates oxidize reduced inorganic sulfer compounds (RISCs) but in addition can oxidize ferrous iron, though autotrophic growth on the latter has not been confirmed.

An acidophilic bacterium isolated from coal mine drainage water in Missouri, USA, differed from other rod-shaped iron oxidizers in apparently being unable to oxidize sulfur (Harrison, 1982). Even so, the isolate (strain "m-1") was considered to be a strain of *Thiobacillus ferrooxidans* (now *Acidithiobacillus ferrooxidans,* see below). Harrison (1982) also showed that strain m-1 had little or no DNA homology with other strains of "*T. ferrooxidans*" and also had a significantly higher G+C content of its chromosomal DNA. Phylogenetic analysis placed isolate m-1 firmly in the (β subgroup of the Proteobacteria (Goebel and Stackebrandt, 1994a) in contrast to an earlier analysis (Lane *et al.,* 1992). A nearly full-length 16S rRNA gene sequence from the m-1 strain deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ) also placed this acidophile in the β subgroup (Fig. 2) and confirms that this isolate is unrelated to all other iron-oxidizing acidophiles (Hallberg and Johnson, unpublished).

c. β/γ-Proteobacteria. The best studied of all acidophiles, *Acidithiobacillus ferrooxidans,* falls within a monophyletic group of bacteria that diverges from other bacteria at the cusp between the β and γ subgroups of the Proteobacteria (see below). Formerly known as *Thiobacillus ferrooxidans,* a reevaluation of the genus *Thiobacillus* by Kelly and Wood (2000), based on comparisons of physiology and 16S rRNA gene sequences, resulted in the reassignment of this and two other *bona fide* acidophilic thiobacilli (*Thiobacillus thiooxidans* and *Thiobacillus caldus*) into the new genus *Acidithiobacillus*. Besides obligate acidophily, these three bacteria share the ability to grow aerobically and autotrophically using sulfur, and other RISCs, as electron donors.

Acidithiobacillus thiooxidans was the very first acidophilic (as defined in this article) prokaryote to be isolated (Waksman and Joffe, 1921). It is an obligately acidophilic and aerobic bacterium, which grows by coupling the oxidation of elemental sulfur and RISCs to the reduction of molecular oxygen. Brock and Gustafson (1976) reported that *Acidithiobacillus thiooxidans* could reduce ferric iron to ferrous, though did not demonstrate that this acidophile could grow via anaerobic respiration using ferric iron. *Acidithiobacillus thiooxidans* is extremely acidophilic, growing between pH 0.5 and 5.5 and with an optimum of pH 2–3. Oxidation of elemental sulfur and RISCs by this (and other) sulfur oxidizers can result in extremely low culture pH, which can be deleterious to the growth of other, less acid-tolerant, microorganisms. *Acidithiobacillus thiooxidans* appears to be obligately autotrophic; organic materials such as yeast extract do not affect growth yields. The type strain (ATCC 19377) and other isolates are also fairly temperature sensitive, and grow poorly, if at all, at 35°C and above.

Those isolates of "*At. thiooxidans*" that were, up to relatively recently, claimed to be thermotolerant (growth at 40°C and above) were almost certainly strains of *At. caldus* (Hallberg and Lindström, 1994). This is a moderately thermophilic acidophile (optimum and upper temperatures for growth 45 and 52°C, respectively, for the type strain but others can grow at higher temperatures) which, like *At. thiooxidans,* grows autotrophically using elemental sulfur and other RISCs as energy source. In contrast to the latter, however, *At. caldus* can grow mixotrophically when provided with yeast extract or glucose. The original isolate (BC13) was obtained from acidic water in a coal spoil heap (Birch Coppice) in central England (Marsh and Norris, 1983), but since then isolates have been obtained in various locations, including geothermal areas, including Yellowstone National Park, Montserrat (West Indies), Iron Mountain, California, and bioleaching operations (heaps and bioreactors) in South Africa and Australia. Mixed culture studies have shown that *At. caldus* is able to out-compete other sulfur-oxidizing acidophiles (including *At. thiooxidans*) even at relatively low (30°C and above) temperatures (Hallberg *et al.,* 2001). *Acidithiobacillus caldus* is a dominant prokaryote in stirred tank cultures leaching mineral ores (e.g., Norris *et al.,* 2000), where it appears to utilize the relatively energy-rich RISCs produced by ferric iron attack on sulfide minerals (Section III.C).

The most significant difference between *At. ferrooxidans* and other *Acidithiobacillus* spp. is the ability of the former to grow autotrophically with ferrous iron as sole energy source. It grows at slightly higher temperatures than *At. thiooxidans* (~37°C) but is less acidophilic (minimum pH 1.3–1.5). Such data concerning *At. ferrooxidans* are quite variable, however, this being generally attributed to "strain variation." Indeed,

many bacterial isolates have been ascribed the binomial "*Acidithiobacillus ferrooxidans*" on the basis that they are iron/sulfur-oxidizing acidophilic autotrophs, but these isolates probably represent two, or even more, different species (e.g., Kelly and Wood, 2000). *Acidithiobacillus ferrooxidans* has been the subject of a great deal of research, and of a number of review articles (e.g., Leduc and Ferroni, 1994), based on the belief that it was the main, or (for many years) the sole, biological agent responsible for catalyzing the oxidative dissolution of acid-stable sulfide minerals (such as pyrite) at low pH, thereby leaching metals from minerals and generating AMD. However, this assumption has been challenged and shown, in many cases, not to be the case. Other iron-oxidizing bacteria (principally *Leptospirillum* spp.) and archaea are actually more prevalent in many industrial and environmental situations.

Acidithiobacillus ferrooxidans is a remarkable organism in many respects. It is, for example, able to tolerate extremely high concentrations of many heavy metals (e.g., >300 m*M* copper, zinc, and iron) though other cations (notably silver) and many anions (other than sulfate) are toxic in quite low concentrations (often <1 m*M*). It is also a highly flexible bacterium in metabolic terms, being able to grow aerobically or anaerobically (using ferric iron as terminal electron acceptor) on a variety of electron donors (ferrous iron, RISCs, hydrogen and formic acid) (Fig. 3).

A fourth *Acidithiobacillus* sp. was recognized by Kelly and Wood (2000). *Acidithiobacillus albertensis* (formerly *Thiobacillus albertis*) was originally isolated from acidic soil adjacent to a sulfur stockpile in Alberta, Canada (Bryant *et al.,* 1983). Physiologically, it resembles

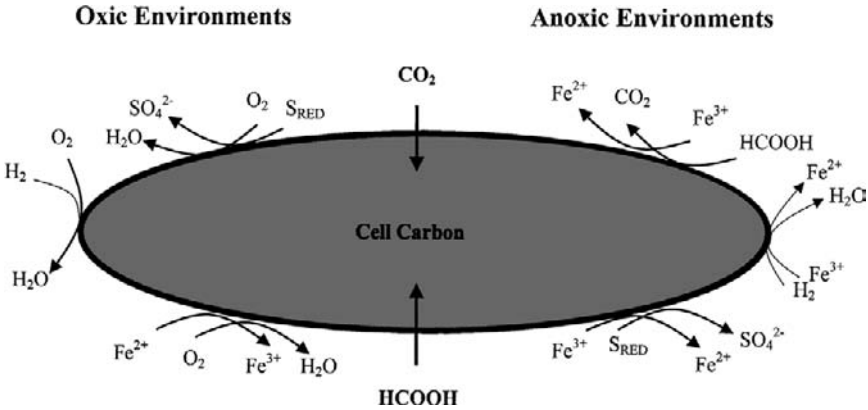

FIG. 3. Metabolic flexibility in the mesophilic acidophile *Acidithiobacillus ferrooxidans* shown as the variety of possible couplings of electron donors and acceptors.

TABLE II

ACIDOPHILIC PROKARYOTIC MICROORGANISMS WITH CHROMOSOMAL G+C CONTENT GIVEN IN PARENTHESIS (AS MOL%)

Gram-negative bacteria	Gram-positive bacteria	Archaea
	Sulfur oxidizers	
H. acidophilus (35)	*Sb. disulfidooxidans* (53)	*S. hakonensis* (38)
A. acidophilus (64)		*S. yangmingensis* (42)
At. thiooxidans (50–52)		
At. caldus (62–64)		
At. albertensis (62)		
Tm. cuprina (66–69)		
	Iron oxidizers	
L. ferrooxidans (51–56)[a]	*Am. ferrooxidans* (67–69)	*Fp. acidiphilum* (37)
"*Fm. acidiphilum*" (52–55)		
	Sulfur/iron oxidizers	
At. ferrooxidans (58–59)	*Sb. thermosulfidooxidans* (48/50)	*Ad. brierleyib*[b] (31)
"*T. prosperus*" (63–64)	*Sb. acidophilus* (55–57)	*Metallosphaera* spp. (45–46)
	"*Sb. montserratensis*" (58)	*S. metallicus* (38)
		Sc. yellowstonensis (45)
	Sulfur-reducing autotrophs/heterotrophs	
		Thermoplasma spp. (38–46)
		Al. aceticus (54)
		Ac. infernus (31)
		Ac. ambivalens (33)
		Ss. ohwakuensis (33)
		Sg. azoricus (31)
	Iron-reducing heterotrophs	
Acidiphilium spp. (63–70)	*Alicyclobacillus* spp. (ND[c])	
	Other heterotrophs	
Acidocella spp. (59–65)		*S. acidocaldarius* (37)
Ab. capsulatum (60)		*S. solfataricus* (34–36)
As. rubrifaciens (69)		*S. shibitae* (35)
Amn. methanolica (63–65)		*Picrophilus* spp. (36)

[a] These data probably include more than one *Leptospirillum* spp.
[b] *Acidianus brierleyi* can also reduce S (see text).
[c] ND: not determined.

At. thiooxidans in being an obligately autotrophic sulfur oxidizer. However, the G+C content of the chromosomal DNA of *At. albertensis* is significantly greater than that of *At. thiooxidans* (Table II), and the two acidophiles were also differentiated originally on the basis of pH range (though this was only tested on solid medium in the case of *At. albertensis*) and the possession of the latter of a glycocalyx and a tuft of polar flagella. The exact phylogenetic relationship of the two species is,

however, unknown, as no 16S rDNA sequence data for *At. albertensis* are apparently available. In addition, the original isolate appears to be lost from culture (Kelly and Wood, 2000).

In the pioneering phylogenetic study on acidophiles (Lane *et al.,* 1992), a group that includes *At. ferrooxidans* and *At. thiooxidans* was shown to diverge from the branch between the β- and γ-Proteobacteria, though it was conceded that analysis based on nearly complete rRNA sequences might lead to a different phylogenetic tree topology. More recently, it has been proposed that these two species, and also *At. caldus,* belong to the γ-Proteobacteria (McDonald *et al.,* 1997). This conclusion, however, is based on a phylogenetic tree that was made without the inclusion of an outgroup (a very distantly related sequence used to provide a "root" to a tree). In our phylogenetic analyses with an outgroup (Fig. 4), we consistently find the acidithiobacilli form a deep branching group within the Proteobacteria, in a location similar to that found in the study by Lane *et al.* (1992). Similar tree topologies have been found by other researchers as well (Goebel and Stackebrandt, 1994a). It may be that these acidophiles belong to yet another group of the Proteobacteria, but we feel that the often used designation "β/γ-Proteobacteria" is more appropriate until further analyses are performed.

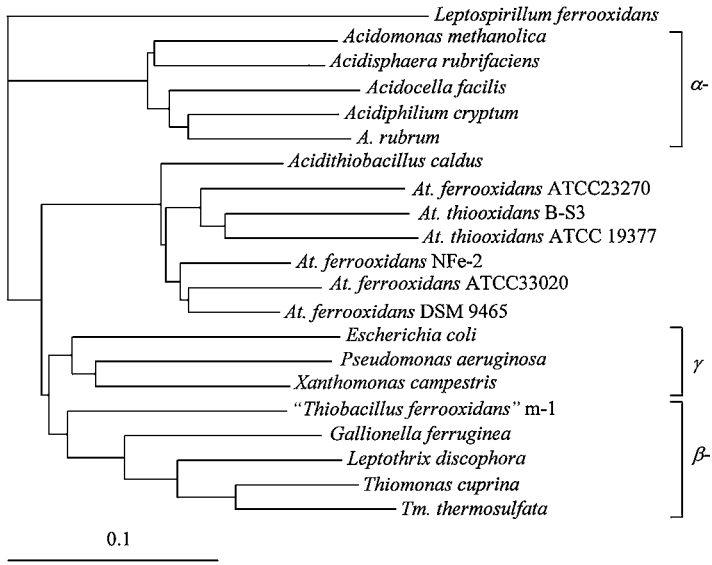

FIG. 4. The phylogenetic grouping of acidithiobacilli and with other *Proteobacteria* based on 16S rRNA sequence data. The 16S rRNA gene sequence of *L. ferrooxidans* was used as outgroup.

5. *Actinobacteria*

Two species of acidophiles (only one of which has been validated at the time of writing) belong to the recently designated bacterial class *Actinobacteria* (Stackebrandt *et al.,* 1997). Both are iron oxidizers, though they differ in their response to temperature.

a. Acidimicrobium ferrooxidans. One of the first moderately thermophilic iron-oxidizing Gram-positive bacteria to be isolated, strain TH3, appeared to be obligately heterotrophic, requiring yeast extract to grow successfully on ferrous iron and to leach sulfide minerals (Norris and Barr, 1985). The rod-shaped isolate grew as characteristic filaments in liquid media, and did not oxidize sulfur. A second strain (coded ICP) was isolated from an Icelandic geothermal site by enrichment in pyrite and shown to be able to fix CO_2, as well as to utilize organic compounds. The Icelandic isolate was selected as the type strain of *Am. ferrooxidans* (Clark and Norris, 1996). Mixed cultures of *Am. ferrooxidans* and *Sulfobacillus* spp. (see below) were more efficient at oxidizing ferrous iron in autotrophic media using normal (atmospheric) concentrations of CO_2 than when these acidophiles were grown in pure culture (Clark and Norris, 1996).

b. "Ferrimicrobium acidiphilum". Mesophilic iron-oxidizing bacteria, which apparently are not able to fix CO_2, have been isolated from mine sites in Wales and the USA (Johnson *et al.,* 1995). These bacteria, provisionally called *Ferrimicrobium acidiphilum,* are closely related to *Am. ferrooxidans,* 16S rRNA gene homology of 95%, and represent the only other cultivated microbes from this group of acidophiles. *Ferrimicrobium acidiphilum* are able to oxidize ferrous iron or pyrite in yeast extract amended liquid media. Mixed cultures of *Fm. acidiphilum* and *At. thiooxidans* or *A. acidophilum* (none of which leach sulfides in pure culture) were shown to accelerate the oxidative dissolution of pyrite, presumably due to the latter providing the iron-oxidizing heterotroph with organic carbon compounds (cell exudates, lysates etc. (Bacelar-Nicolau and Johnson, 1999). Like *Am. ferrooxidans, Fm. acidiphilum* appears unable to oxidize sulfur or form endospores. These organisms grow as single or paired motile rods, and may form filaments when stressed.

c. Uncultivated Members of Actinobacteria. A group of environmental clones from the Iron Mountain site (Bond *et al.,* 2000b) have been identified that belong to the Actinobacteria division. One of the clones has an identical 16S rRNA gene sequence to *Fm. acidiphilum,* one is very

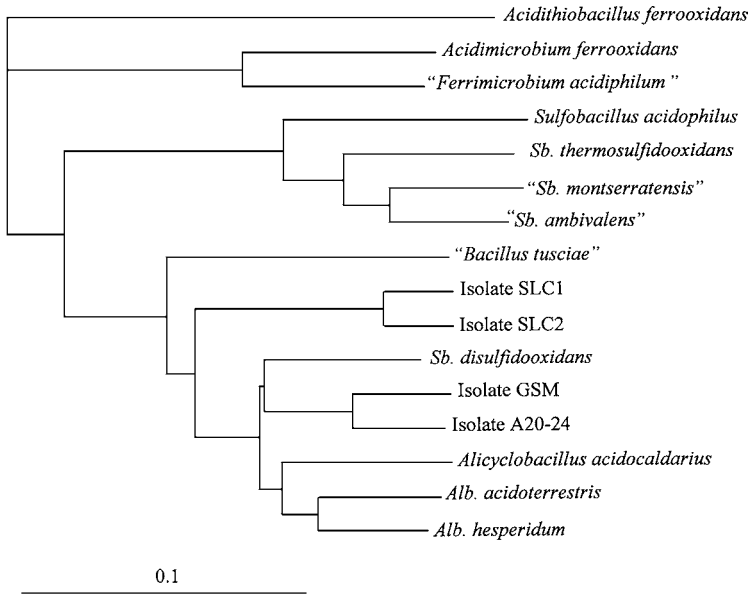

FIG. 5. 16S rRNA phylogenetic tree of Gram-positive acidophiles. This tree was rooted with *At. ferrooxidans* as outgroup.

related to *Am. ferrooxidans,* and one is only distantly related. Except for the highly related clones, it is difficult to draw any inferences as to what role these microbes play at the site solely on the basis of phylogenetic analysis.

6. *Low G+C Gram-Positive Bacteria*

Currently, there are two recognized acidophilic genera located in the "Low G+C Gram-positive" division of the domain Bacteria, though at least three other phylogenetically distinct groups exist (see Fig. 5).

a. Genus Sulfobacillus. Both currently recognized species of *Sulfobacillus* are endospore-forming moderate thermophiles, typically growing between 40 and 60°C. These acidophiles have been isolated from geothermal areas (e.g., Iceland, Yellowstone, Montserrat), mineral spoil heaps, and biomining operations. *Sulfobacillus* spp. may grow autotrophically, using ferrous iron, sulfur and RISCs, and sulfide minerals as sources of energy, though their propensities for CO_2 fixation tend to be rather limited (Norris *et al.,* 1996). They may also grow mixotrophically (e.g., in ferrous iron/yeast extract medium) or heterotrophically (e.g., using glucose as carbon and energy source). *Sulfobacillus thermosulfidooxidans* (the first species to be described) and *Sb. acidophilus* may

be differentiated from each other on the basis of G+C content (Table II). In addition, some strains of *Sb. thermosulfidooxidans* grow as coryneforms, whereas *Sb. acidophilus* generally grow as regular rods, though chains of distorted cells have been observed under some growth conditions (Norris *et al.,* 1996). In contrast to early reports, *Sulfobacillus* spp. are facultative anaerobes, able to grow in anoxic media using ferric iron as electron acceptor and either an organic (e.g., glycerol) or inorganic (e.g., tetrathionate) electron donor (Bridge and Johnson, 1998).

An apparently novel "*Sulfobacillus*" sp. (*Sb. disulfidooxidans*) was described by Dufresne *et al.* (1996). This is a mesophilic aerobic bacterium which, in contrast to the species described above, does not oxidize ferrous iron though it can utilize RISCs and organic substrates as energy sources. Unfortunately, the taxonomic affiliation of *Sb. disulfidooxidans* was based on an erroneous 16S rRNA sequence having been deposited in the data bank for *Sb. thermosulfidooxidans*. This error has since been amended, and it is now clear that *Sb. disulfidooxidans* is more closely related to *Alicyclobacillus* spp. than to *Sulfobacillus* spp. (Fig. 5), and as such, requires reclassification. The presence of the diagnostic ω-alicyclic fatty acid (Section II.A.6.b) in *Sb. disulfidooxidans,* in addition to 16S rRNA phylogeny, justifies the reclassification of this microbe as "*Alicyclobacillus disulfidooxidans*".

Sulfobacillus-like bacteria were isolated from a number of locations on the Caribbean island of Montserrat, just prior to the active period of volcanism in the late 1990s (Atkinson *et al.,* 2000). The Montserrat isolates exhibit about 97% 16S rDNA homology with each other and with *Sb. thermosulfidooxidans* and only about 90% with *Sb. acidophilus* (Fig. 5). In contrast to known *Sulfobacillus* spp., the new isolates were mesophilic (Yahya *et al.,* 1999). These isolates had many physiological characteristics in common with *S. acidophilus* and *Sb. thermosulfidooxidans*. They were far more tolerant of extreme acidity, oxidizing ferrous iron in media poised at pH 0.7, thus making them the most acidophilic of all iron-oxidizing bacteria yet described. Two names have been proposed for these microbes, "*Sb. montserratensis*" and "*Sb. ambivalens*".

b. Alicyclobacillus *spp.* Alicyclobacilli are spore-forming, obligately heterotrophic rod-shaped moderately thermophilic acidophiles. Four species are currently recognized: *Alb. acidocaldarius, Alb. acidoterrestris,* Alb. *cycloheptanicus,* and *Alb. hesperidum* (Fig. 5). One characteristic diagnostic feature of bacteria belonging to this genus is the possession of ω-alicyclic fatty acid as the major lipid component in their cell membranes (Wisotzkey *et al.,* 1992). Novel *Alicyclobacillus*-like isolates (which also produce ω-alicyclic fatty acid) have been obtained from geothermal sites in Yellowstone National Park (Johnson *et al.,* 2001b).

These are more acid tolerant than the four classified species, growing at pH 2.0 and above. These isolates can reduce ferric iron, though whether this can support growth in anoxic media has not been established. There are no reports of *Alicyclobacillus*-like bacteria being isolated from mineral leaching operations, though the Yellowstone isolates have been successfully maintained in mixed cultures with *Sulfobacillus* spp. in laboratory-scale pyrite cultures (D. B. Johnson *et al.,* unpublished data).

c. Miscellaneous/Unclassified Gram-Positive Species. Two further groups of phylogenetically distinct mineral-oxidizing bacteria that are located in the Low G+C Gram-positive division have recently been isolated (Fig. 5). One group (the "SLC series") is mesophilic, obligately heterotrophic iron oxidizers which have been isolated from sulfidic regoliths subjected to accelerated weathering under laboratory conditions (Johnson *et al.,* 2001a). The other group is represented by an isolate (a moderate thermophile designated GSM) from mine spoil at the Golden Sunlight mine, Montana (Johnson *et al.,* 2001a), and 3 halotolerant iron-oxidizing isolates from Sydney harbour (Holden *et al.,* 2001).

A final acidophile in this phylogenetic cluster group isolated from a solfatara field is called "*Bacillus tusciae*" (Bonjour and Aragno, 1984). Apart from its isolation and phylogeny, little is known about this acidophile. The acidophiles in the Low G+G Gram-positive division have great metabolic diversity relative to other acidophiles, reflecting the close phylogenetic relationship to bacteria of the metabolically diverse genus *Bacillus*. It appears that the biodiversity of Gram-positive acidophiles is probably considerably greater than has been recognized hitherto.

7. *Unclassified Acidophilic Bacteria*

A number of other acidophilic bacteria have been described, and in some cases, designated species names. Without confirmation of their phylogenetic affiliations, they are listed here.

"*Thiobacillus prosperus*" is a halophilic (growing in up to 6% sodium chloride) chemolithotrophic acidophile. All strains were isolated from a geothermally heated seafloor of the coast of Italy (Huber and Stetter, 1989). These strains grow by oxidation of a variety of sulfide minerals (including pyrite, chalcopyrite, and galena), but somewhat curiously, poorly on elemental sulfur or ferrous iron, and they are obligately autotrophic. Cells are larger (3–4 μm long) than *At. ferrooxidans,* and are also distinguished by a higher maximum temperature for growth (41°C) and mol% G+C of their chromosomal DNA (Table II). Kelly and Wood (2000) chose not to reclassify *T. prosperus* in a revision of the genus *Thiobacillus* due to insufficient (principally 16S rDNA sequence) data.

Other acidophilic bacteria that have been described in the literature, though have not been ascribed species names, include 3 iron-oxidizing

mesophiles: T3.2 (de Siloniz *et al.,* 1993), Funis (Blake *et al.,* 1993), and isolate CCH7 (Johnson *et al.,* 1992), an obligately heterotrophic isolate that grew as long filaments, reminiscent of the neutrophilic iron oxidizer *Leptothrix*.

B. Archaea

Acidophilic archaea occur in the Euryarchaeota and Crenarchaeota; the third archaeon kingdom (the Korarchaeota) does not currently contain any cultivated representatives.

1. *Acidophilic Euryarchaeota*

Acidophilic Euryarchaeota belong to the order *Thermoplasmales* and comprise three families: the *Thermoplasmaceae,* the *Picrophilaceae,* and the *Ferroplasmaceae,* each of which is currently represented by a single recognized genus (Fig. 6).

a. Thermoplasma *spp. Thermoplasma acidophilum* was one of the first acidophilic heterotrophic prokaryotes to be isolated (from a coal refuse pile) and characterized (Darland *et al.,* 1970). This archaeon has also been isolated from solfatara fields in different global locations,

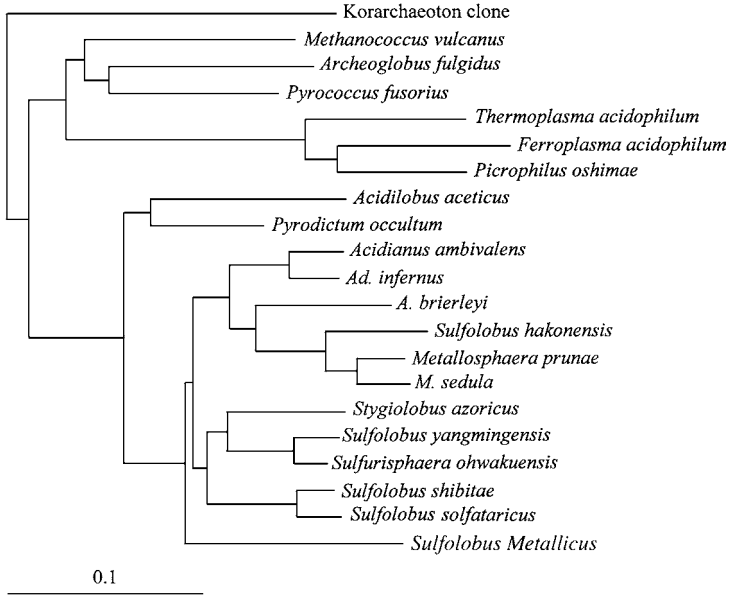

Fig. 6. Phylogenetic tree, based on 16S rRNA gene sequence, showing the relationship of selected archaea to acidophilic archaea.

along with a second species, *Tp. volcanium* (Segerer *et al.,* 1988). *Thermoplasma acidophilum* and *Tp. volcanium* were differentiated on the basis of some physiological properties, G+C contents of chromosomal DNA, and DNA homologies.

Thermoplasma spp. lack cell walls and are mostly motile. Various cellular morphologies have been observed, including filaments, disks and club shapes, though cocci tend to dominate (Segerer *et al.,* 1988). Both species are moderately thermophilic, growing optimally at 59–60°C, and at a maximum of 62°C (*Tp. acidophilum*) and 67°C (*Tp. volcanium*), and with pH minima of ~1.0. *Thermoplasma* spp. are obligate heterotrophs, and do not oxidize ferrous iron or RISCs. They are facultative anaerobes, able to grow in anoxic cultures by sulfur respiration, wherein elemental sulfur (as terminal electron acceptor) is reduced to hydrogen sulfide.

b. Picrophilus *spp.* Hyperacidophilic, moderately thermophilic (temperature optimum 60°C) archaea isolated from solfataric hydrothermal areas in Japan were found to be phylogenetically distinct from *Thermoplasma* spp. (Schleper *et al.,* 1995). Two separate species were identified: *P. oshimae* and *P. torridus*. Both grew as irregular cocci, and in contrast to *Thermoplasma* spp., both *Picrophilus* spp. possess a surface layer, though its structure appears to be different to that of other archaea. As with *Thermoplasma* spp., *Picrophilus* spp. are obligately heterotrophic, though the latter are strict aerobes. One other distinguishing feature is the extreme tolerance of *Picrophilus* spp. to proton acidity; the pH optima for growth of both species is about 0.7, and growth occurs in media poised as low as pH 0.

c. Ferroplasma acidiphilum. The type strain of *Fp. acidiphilum* was isolated from a pilot plant bioreactor leaching an arsenopyrite/pyrite concentrate in Kazakhstan (Golyshina *et al.,* 2000). Cells are pleomorphic and lack a cell wall. In contrast to other acidophilic Euryarchaeota, *Fp. acidiphilum* oxidizes ferrous iron (but not sulfur) and does not appear to grow in anoxic environments. Golyshina *et al.* (2000) claimed that this archaeon is an obligate autotroph though it did not grow in ferrous iron liquid media unless supplemented with yeast extract. Also in contrast to *Thermoplasma* and *Picrophilus* spp., *Fp. acidophilum* is mesophilic, growing optimally at about 33°C, with an upper limit of 45°C. Its pH optimum for growth is 1.7, and the lower limit for growth pH 1.3.

Other archaea, apparently very closely related to *Fp. acidiphilum,* have also been found in mineral leaching environments. The 16S rRNA gene of an archaeon was obtained from a chalcopyrite leaching operation

in Chile (Vasquez *et al.,* 1999) that is 97% homologous to the gene from *Fp. acidiphilum*. Given that, out of 912 bases sequenced, two gaps were present and all but 2 of the 23 other mismatches with the *Fp. acidiphilum* sequence consisted of unknown nucleotides, this archaeon could be an isolate of *Fp. acidiphilum*. Unfortunately, the authors were unable to obtain the archaeon in pure culture for further studies. Another archaeon isolated from the Iron Mountain site was shown to have a nearly identical 16S rRNA gene sequence (843 identical bases out of 849 bases, with 3 gaps and 3 unknown nucleotides) to that of *Fp. acidiphilum* (Edwards *et al.,* 2000b). Several phenotypic differences from *Fp. acidiphilum* were noted in that study and the Iron Mountain isolate was named "*Fp. acidarmanus*". Further comparative studies are required, however, before this new species name can be accepted as the physiological differences used to distinguish between the two isolates can be due solely to strain variation (there was only a single isolate used in each of the above studies) or could be due to the use of different culture media (or both).

2. *Acidophilic Crenarchaeota*

The first acidophilic archaeon to be isolated and characterized was a *Sulfolobus* sp. (*S. acidocaldarius*), which subsequently gave its name to an order within the Crenarchaeota kingdom. The order *Sulfolobales* (Burggraf *et al.,* 1997) includes aerobic and facultatively anaerobic acidophiles, some of which are able to oxidize (and reduce) sulfur (Fig. 6). All acidophilic Crenarchaeota thus far isolated are thermophilic, growing at $>60^{\circ}$C.

a. Sulfolobus *spp.* *Sulfolobus* spp. are the most numerous of all currently recognized acidophilic archaea. There was some confusion about the metabolic abilities of *S. acidocaldarius* and *S. solfataricus,* probably because some of the earliest cultures were not pure (Grogan, 1989). Neither of these archaea are now considered to oxidize sulfur, and both are obligate heterotrophs (Norris and Johnson, 1998). As with other *Sulfolobus* spp., cells are coccoid, though they may appear lobed or irregular. *Sulfolobus solfataricus* is more tolerant of high temperatures (optimum 87°C) though it is less acidophilic (pH optimum $\sim$ 4.5) than some other species.

A likely contaminant microorganism which led to erroneous conclusions concerning the metabolic capabilities of *S. acidocaldarius* and *S. solfataricus,* is another *Sulfolobus* sp., *S. metallicus* (Huber and Stetter, 1991). This is an obligately autotrophic acidophile which grows by oxidizing elemental sulfur, RISCs, ferrous iron, or sulfide ores. This prokaryote, together with *Metallosphaera* spp. (see Sec. II.B.2.c),

are probably the most significant mineral-oxidizing microorganisms at >60°C (Norris *et al.,* 2000).

Two other species of *Sulfolobus* have been described. *Sulfolobus hakonensis* (Takayanagi *et al.,* 1996) is an aerobic facultative chemolithotroph, though growth is poor in organic media. *Sulfolobus yangmingensis* was isolated from a geothermal vent in Taiwan (Ren-Long *et al.,* 1999). It is a facultative chemolithotroph, utilizing elemental sulfur, RISCs, or various organic materials as sources of energy. The G+C content of its genomic DNA is also greater than other *Sulfolobus* spp.

b. Acidianus *spp.* The genus *Acidianus* derives its name from the Roman god Janus, the god of doors or gates, who is often portrayed with two faces looking in opposite directions. This choice of generic name derives from the ability of *Acidianus* spp. to use sulfur either as an electron donor when grown aerobically, or as an electron acceptor in anaerobic cultures (Segerer *et al.,* 1986). There are currently three recognized species of *Acidianus*: *Ac. infernus, Ac. brierleyi* (formerly *S. brierleyi*), and *Ac. ambivalens* (formerly *Desulfurolobus ambivalens*) (Fuchs *et al.,* 1996a). These species may be differentiated on various molecular (e.g., DNA homologies, 16S rDNA sequences) and physiological traits. *Acidianus infernus* and *Ac. ambivalens* are both obligately chemolithotrophic sulfur-oxidizer/reducers. *Acidianus brierleyi* can grow autotrophically (oxidizing ferrous iron, as well as sulfur) or heterotrophically in various complex organic media.

c. Metallosphaera *spp.* Two species of *Metallosphaera* are currently recognized: *M. sedula* (Huber *et al.,* 1989) and *M. prunae* (Fuchs *et al.,* 1996b). Both are aerobic, facultative chemolithotrophs, oxidizing sulfur, sulfide minerals, and hydrogen, or growing on complex organic substrates, such as yeast extract. *Metallosphaera sedula* is able to solubilize metal sulfides at slightly higher temperatures (~80°C) than *S. metallicus,* and may have greater potential for high temperature mineral bioleaching.

d. Sulfurococcus yellowstonensis. Karavaiko *et al.* (1994) isolated a facultatively autotrophic *Sulfolobus*-like archaeon from a geothermal pool in Yellowstone National Park. The isolate, originally named *Sulfurococcus yellowstonii* (Karavaiko *et al.,* 1994), was able to oxidize ferrous iron and sulfide minerals, as well as reduced sulfur. The structure of its 5S rRNA was ascertained and showed that *Sc. yellowstonensis* groups within the order *Sulfolobales,* but there are no sequence data available on its 16S rRNA gene.

e. Sulfurisphaera ohwakuensis. *Sulfurisphaera ohwakuensis* was isolated from acidic hot springs in Japan (Kurosawa *et al.,* 1998). This archaeon has a number of physiological traits in common with *Acidianus* spp. (e.g., in being a facultative anaerobe), though it is more closely related (though phylogenetically distinct) to *Stygiolobus azoricus* (described below). *S. ohwakuensis* is one of the most thermotolerant acidophiles, growing at up to 92°C, with a temperature optimum of 84°C.

f. Stygiolobus azoricus. *Stygiolobus azoricus* was the first obligately anaerobic thermoacidophile to be isolated (Segerer *et al.,* 1991), originally from San Miguel island in the Azores. This archaeon grows by coupling the oxidation of hydrogen to the reduction of elemental sulfur (forming hydrogen sulfide).

g. Acidilobus aceticus. A second obligately anaerobic thermoacidophilic archaeon has recently been described, *Al. aceticus* (Prokofeva *et al.,* 2000). In contrast to *Sg. azoricus, Al. azoricus* grows by fermenting starch (forming acetate as a major product) and other complex organic substrates. Growth is stimulated by the addition of sulfur, which is reduced to hydrogen sulfide. Although sharing a similar cell morphology (regular and irregular cocci) with the *Sulfolobales* genera, 16S rDNA sequence data showed it formed a distinct and separate branch within the Crenarchaeota kingdom (see Fig. 6).

III. Metabolism of Acidophiles

As described in the previous section, the metabolic capacity of acidophiles is very diverse. In this section, we will describe in more detail the biochemistry of some of the important metabolic pathways. One important aspect of physiology of the biomining bacteria in terms of energy demand is the fixation of carbon dioxide. Most acidophiles that are important to biomining (and AMD generation) are autotrophic, which means that they derive the majority of the cellular carbon via fixation of carbon dioxide. The carbon dioxide fixation requires reducing equivalents, usually in the form of NAD(P)H. Since the growth substrates of the acidophiles are not sufficiently reduced to generate reducing equivalents, they must reverse the electron flow from the direction oxygen (or other terminal electron acceptor), and thus the generation of ATP, to the NADP-oxidoreductase (Ingledew, 1982). The first direct evidence of this happening in *At. ferrooxidans* has recently been provided (Elbehti *et al.,* 2000).

A. Iron Metabolism

One of the key activities of acidophiles is the oxidation of ferrous iron to ferric iron. The reverse reaction, which is the reduction of ferric iron to ferrous, is also important in acidophilic environments. The Fe^{2+}/Fe^{3+} redox couple has a standard reduction potential (E_0) of +770 mV (at pH 2), which is nearly equivalent to that of oxygen. Thus ferric iron can also serve as a terminal electron acceptor, yielding nearly as much energy as oxygen.

1. *Ferrous Iron Oxidation*

As with many other aspects of the microbiology of acidophiles, the most thorough studies into the oxidation of ferrous iron has been carried out with *At. ferrooxidans*. The biochemistry of iron oxidation has been the subject of a recent review (Blake and Johnson, 2000), and thus will not be explored here in depth. As mentioned above, the redox couple of ferrous to ferric iron is sufficiently high that only molecular oxygen can serve as electron acceptor for acidophilic microorganisms. The free energy associated with the oxidation reaction [Eq. (1)] is rather small, -30 kJ mol^{-1} at 30°C, but there is usually a large abundance of ferrous iron in acidic environments relative to other potential electron donors.

The oxidation of ferrous iron is a proton-consuming reaction [Eq. (1)] and is believed to occur outside the cell (as the product, ferric iron, is highly insoluble at the pH of the cytoplasm of microorganisms—including acidophiles).

$$4Fe^{2+} + O_2 + 4H^+ \rightarrow 4Fe^{3+} + 2H_2O. \quad (1)$$

The consumption of protons in the cytoplasm creates the necessary proton gradient to generate ATP. The electrons liberated from iron can be shuttled in a "reverse" direction for the generation of reducing power. This process is generally assumed to occur in all acidophiles that oxidize ferrous iron, though direct evidence has not been provided, except in the case of *At. ferrooxidans* (as noted above).

The enzymology of ferrous iron oxidation has also been most thoroughly studied in *At. ferrooxidans* (Blake and Johnson, 2000). The components of the electron transport chain are not fully resolved, but include a periplasmic protein called rusticyanin (Cox and Boxer, 1986), an acid stable c-type cytochrome that catalyzes the reduction of rusticyanin in the presence of ferrous iron (Blake and Shute, 1994), and cytochrome c/oxygen reductase. Recently, the genes from *At. ferrooxidans* that encode these components have been cloned and sequenced (Appia-Ayme *et al.,* 1999). They are co-transcribed and expressed to

much higher levels in iron-grown cells than in sulfur-grown cells, indicating that they are all involved in the oxidation of ferrous iron. Another set of genes, called *resB* and *resC,* was cloned from a mutant of *At. ferrooxidans* that had lost the capacity to oxidize iron (Cabrejos *et al.,* 1999). The genes were shown, by homology, to encode for proteins that are involved in the maturation of cytochromes. It was also reported that the two genes were of the proposed system II cytochrome biogenesis pathway, while γ-Proteobacteria were reported to use system I. The authors concluded that these genes could have arisen in *At. ferrooxidans* by lateral gene transfer, or it could point to the fact that this acidophile is difficult to place into one of the currently recognized phylogenetic groups within the Proteobacteria (see discussion in Sec. II.A.4.c).

In contrast to the well-studied enzymology of ferrous iron oxidation by *At. ferrooxidans*, very little is known about the enzymology of other ferrous iron oxidizing acidophiles. An acid stable cytochrome that is slowly oxidized by ferrous iron was found in extracts of ferrous iron grown *L. ferrooxidans* (Barr *et al.,* 1990). No rusticyanin was found in this bacterium. The Gram-positive iron oxidizing strains TH1 and ALV (now recognized as *Sb. thermosulfidooxidans* and *Sb. acidophilus*, respectively) had similar spectra, suggesting that they have similar electron chain components. Interestingly, no low molecular weight electron carrier was observed in these strains, probably reflecting the lack of a periplasmic space. Finally, in a survey of iron-oxidizing acidophilic archaea, members of three different genera exhibited the same spectral characteristics, indicating that they share similar electron transport chain components (Barr *et al.,* 1990). Similar data were found by another group in a separate study (Blake *et al.,* 1993).

2. *Ferric Iron Reduction*

Ferric iron occurs in high concentrations in many extremely acidic environments, due in part to the activity of iron-oxidizing microbes coupled with the high solubility of ferric iron below pH 2, and could, in theory, easily serve as an electron acceptor for acidophiles. One of the earliest studies on the reduction of ferric iron by acidophiles showed *At. ferrooxidans* was able to couple the oxidation of sulfur to ferric iron reduction under anaerobic conditions (to prevent the oxidation of the ferrous iron generated) (Brock and Gustafson, 1976). In that same study, it was shown that a culture of *S. acidocaldarius* (probably not that archaeon, but rather a contaminant—see Sec. II.B.2.a) could also couple sulfur oxidation to the reduction of ferric iron under anaerobic conditions, while *At. thiooxidans* could do so only in the presence of oxygen. Growth of the acidophiles was not determined.

Later, it was shown that energy for the uptake of an amino acid by *At. ferrooxidans* could be generated by the oxidation of sulfur coupled to the reduction of ferric iron (Pronk *et al.,* 1991a). *At. ferrooxidans* could also grow under these conditions (Das *et al.,* 1992; Pronk *et al.,* 1992). A recent survey of the other two acidithiobacilli, *At. thiooxidans* and *At. caldus*, has shown that these two sulfur oxidizers are unable to catalyze the reduction of ferric iron anaerobically (Hallberg *et al.,* 2001). In that same study, it was shown that *At. ferrooxidans* could grow by the reduction of ferric iron with tetrathionate as substrate. Similarly, H_2-coupled ferric iron reduction allows the growth of *At. ferrooxidans* (Ohmura *et al.,* 1999).

Heterotrophic acidophiles, mainly of the genus *Acidiphilium,* could also catalyze the reduction of ferric iron (Johnson and McGinness, 1991). These microbes reduced ferric iron even in the presence of oxygen, though the propensity for reduction was greater at low concentrations of oxygen. Recently an acidophilic isolate, shown to be *A. cryptum* by 16S rRNA gene sequence analysis, was found to be able to grow anaerobically by coupling the oxidation of many organic compounds to the reduction of ferric iron (Kusel *et al.,* 1999). This isolate was also able to couple the oxidation of molecular hydrogen to ferric iron reduction. Attempts to detect ferric iron reduction and growth of the sulfur-oxidizing *A. acidophilum* with elemental sulfur or tetrathionate (as would be expected of this *Acidiphilium* species that is able to oxidize RISCs and also reduce ferric iron) have met with limited success (Hallberg *et al.,* 2001).

The biochemistry of ferric iron reduction is not very well understood. Early work with *At. ferrooxidans* showed a respiratory-chain poison inhibited anaerobic oxidation of sulfur with ferric iron. An observation that sulfur-dependent ferric iron reduction and ferrous iron dependent oxygen consumption are both inhibited when *At. ferrooxidans* was grown in a thiosulfate-limited chemostat led to the conclusion that a single oxidoreductase was involved in the oxidation and reduction of iron (Pronk *et al.,* 1992). Alternatively, growth in the thiosulfate-limited chemostat repressed sulfur oxidation by *At. ferrooxidans*. The single oxidoreductase theory, however, appears more likely following the observation that rusticyanin is present in anaerobically grown *At. ferrooxidans* with hydrogen as electron donor (Ohmura *et al.,* 1999).

B. Reduced Inorganic Sulfur Compound Oxidation

RISCs occur naturally in acidic environments (generally around areas of high geothermal activity) and also occur due to the oxidative dissolution of mineral sulfides (see Sec. III.C). RISCs are important electron donors for acidophiles as they offer more electrons per mol substrate

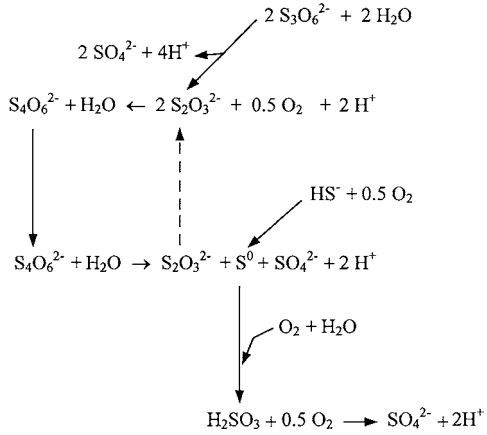

FIG. 7. Schematic diagram showing the sequential reactions during the oxidation of RISCs to sulfate.

compared to ferrous iron. Some electrons enter the electron transport chain at cytochrome b, which allows for more proton translocation and thus the production of more ATP than if the electrons entered at cytochrome *c* (Kelly, 1999). The oxidation of RISCs by microorganisms in general has been the subject of recent reviews (Friedrich, 1998; Kelly *et al.,* 1997).

Early work on the oxidation of RISCs with acidophiles often involved the use of whole cells. Studies with *At. ferrooxidans* (reviewed in Pronk *et al.,* 1990) and with *A. acidophilum* (known then as *Thiobacillus acidophilus*) (Meulenberg *et al.,* 1992b) has led to a proposed path of RISC oxidation where tetrathionate and thiosulfate are intermediates during the oxidation of each (Fig. 7). More recently, the same sequence of reactions for the oxidation of RISCs was proposed for *At. thiooxidans* (Chan and Suzuki, 1994) and *At. caldus* (Hallberg *et al.,* 1996). Interestingly, the conversion of thiosulfate to tetrathionate, and the subsequent oxidation of tetrathionate via thiosulfate, was also found to occur in *Sulfolobus* strain LM (Nixon and Norris, 1992).

The actual mechanism of many of these reactions is not clear. To help resolve the mechanism of RISC oxidation, enzyme purification studies were undertaken initially with *A. acidophilum* because it can grow to high cell densities in chemostat culture with glucose and thiosulfate. Two similar enzymes were purified from *A. acidophilum*—one, a trithionate dehydrogenase, catalyzed the hydrolysis of trithionate to thiosulfate and sulfate (Meulenberg *et al.,* 1992a). The second enzyme, a "thiosulfate dehydrogenase," catalyzed the oxidation of 2 mol thiosulfate to 1 mol tetrathionate (Meulenberg *et al.,* 1993a). A similar enzyme

was partially purified from thiosulfate-grown *At. thiooxidans* (Chan and Suzuki, 1994).

Each of these three enzymes exhibited highest activity at a comparatively low pH, suggesting a periplasmic location for these two steps in the RISC oxidation pathway. A similar conclusion was drawn for the tetrathionate hydrolases that were purified from *A. acidophilum* (de Jong *et al.,* 1997a; Meulenberg *et al.,* 1993b) and *At. ferrooxidans* (de Jong *et al.,* 1997b). This is in contrast to studies performed with whole cells of *At. caldus*, where tetrathionate metabolism was shown to occur in the cytoplasm (Hallberg *et al.,* 1996).

Many of the extremely thermophilic archaea can grow by oxidation of RISCs, but aside from assessment of this property little is known about the mechanisms involved. *Acidianus brierleyi* (*S. brierleyi* at the time) contains a sulfur oxygenase, which was purified from cells grown aerobically in sulfur (Emmel *et al.,* 1986). The enzyme catalyzed the formation of sulfite and traces of thiosulfate, and incorporates molecular oxygen into the sulfite. A sulfur oxygenase-reductase (SOR), purified from the soluble fraction of *A. ambivalens* (Kletzin, 1989), produced sulfite, thiosulfate, and sulfate. The enzyme could simultaneously oxidize and reduce sulfur. The gene encoding the SOR was later cloned and shown to be highly expressed only when *A. ambivalens* was grown aerobically (Kletzin, 1992). Recently, the SOR-encoding gene from another *Acidianus* isolate was cloned and expressed in *Escherichia coli* (He *et al.,* 2000).

C. Metabolism of Metal Sulfides

A wide range of metals, including many of commercial importance (copper, lead, zinc, etc.) occurs as sulfides. Some other metals may be found intimately associated with sulfides in ore bodies, including uranium (as UO_2) and gold (as fine-grain metal). Of all sulfides, the most abundant in the lithosphere is pyrite (FeS_2), which is often found as valueless (gangue) mineral in sulfide ore deposits. Many of the acidophilic prokaryotes described in Section II are able to accelerate the dissolution of sulfide minerals, either by producing strong mineral acid (sulfuric acid), or by generating a powerful oxidizing agent (ferric iron), or both.

Sulfide minerals may be divided into those, such as zinc sulfide (sphalerite), that are acid soluble, and those, such as pyrite and arsenopyrite, that are acid-insoluble. Two different routes (the "thiosulfate" and "polythionate" mechanisms) have been proposed for the biological oxidation of these sulfide mineral types (Schippers and Sand, 1999). Acid-soluble sulfides are readily degraded by sulfur-oxidizing acidophiles, such as *At. thiooxidans* (Garcia *et al.,* 1995). In this model, the mineral is first subjected to proton-mediated dissolution, forming the free metal

and hydrogen sulfide:

$$MS + 2\,H^+ \rightarrow M^{2+} + H_2S. \quad (2)$$

The hydrogen sulfide so formed is microbially oxidized to sulfuric acid, allowing the process to continue:

$$H_2S + 2\,O_2 \rightarrow H_2SO_4. \quad (3)$$

Acid-soluble sulfides may also be attacked by ferric iron [Eq. (4)], producing ferrous iron and polysulfide; the latter may be further oxidized by ferric iron [Eq. (5)], producing elemental sulfur which, in turn, is oxidized to sulfuric acid [Eq. (6)], which will further accelerate mineral dissolution via proton attack [Eq. (2)]:

$$MS + Fe^{3+} + H^+ \rightarrow M^{2+} + 0.5\,H_2S_n + Fe^{2+}. \quad (4)$$

$$0.5\,H_2S_n + Fe^{3+} \rightarrow 0.125\,S_8 + Fe^{2+} + H^+. \quad (5)$$

$$0.125\,S_8 + 1.5\,O_2 + H_2O \rightarrow 2H^+ + SO_4^{2-}. \quad (6)$$

In contrast, sulfides that are resistant to proton attack are oxidized solely by ferric iron, producing thiosulfate as an initial by-product:

$$FeS_2 + 6\,Fe^{3+} + 3\,H_2O \rightarrow 7\,Fe^{2+} + S_2O_3^{2-} + 6\,H^+. \quad (7)$$

Thiosulfate is unstable in acidic liquors, particularly in the presence of ferric iron, and is oxidized to tetrathionate, which in turn decomposes to a variety of other RISCs, sulfur, and sulfate, via a proposed highly reactive disulfane-monosulfonic acid intermediate (Schippers *et al.,* 1996). Importantly for chemolithotrophic acidophiles, both the "polysulfide" and "thiosulfate" mechanisms generate potential inorganic electron donors in the forms of ferrous iron, RISCs and elemental sulfur.

For pyrite, the oxidation state of iron is +2 and that of sulfur is −1. For each mole of FeS_2 oxidized fully to ferric iron and sulfate, only one mol electron is derived from ferrous iron, and 14 mol electrons from the sulfur moiety. The implication is that, even with acid-insoluble sulfides for which iron-oxidizing acidophiles are the key microorganisms in that they mediate the prime attack on the mineral by producing ferric iron, sulfur-oxidizing acidophiles that oxidize the more energy-rich RISC intermediates produced (Sec. III.B) are likely to be abundant and even numerically dominant. This has been borne out recently by the detection of *At. caldus* as the most numerous microorganism in some stirred tank ore-leaching bioreactors (Norris *et al.,* 2000; Rawlings *et al.,* 1999b), even though it is unable to oxidize acid-insoluble sulfides in pure culture. Conversely, that ferrous iron can be regenerated in pyrite leaching cultures either by reacting with sulfide minerals or with RISC

intermediates allows growth yields of *L. ferrooxidans* (which cannot oxidize RISCs) on pyrite to be greater than that possible from the oxidation of ferrous iron present in the mineral alone.

Previously, there has been considerable debate about the proposed "direct" and "indirect" mechanisms of sulfide mineral oxidation, the former referring to that mediated by prokaryotes attached to the mineral surface and the latter to that by free-swimming (planktonic phase) microorganisms. These models have largely been superseded by recent advances in the understanding of how lithotrophic ("rock-eating") acidophiles accelerate the oxidative dissolution of sulfide minerals, as just discussed. For example, acid-insoluble sulfides are attacked by ferric iron produced by iron-oxidizing bacteria and archaea whether they are attached to the mineral surface (in which case the iron is tightly associated with the glycocalyx produced by the sessile cells) or in the liquid phase, where ferric iron oxidizes the mineral following transport by diffusion of mass action, as illustrated in Figure 8. The terms "direct" and "indirect," which used to imply different, but in the case of the former, a largely unknown mechanism, are now redundant and should be replaced with alternatives. More appropriate alternatives may be "contact" and "non-contact" leaching to indicate that the mechanism

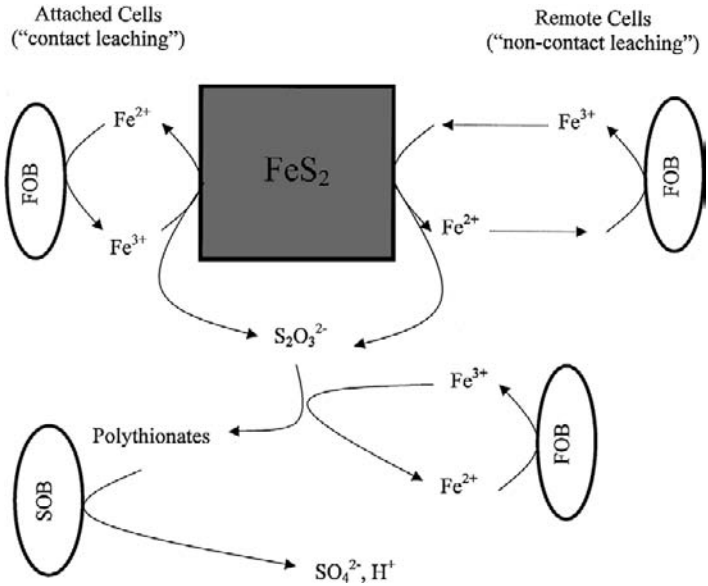

FIG. 8. Hypothetical scheme for the oxidative dissolution of pyrite (FeS_2) by acidophilic bacteria, either as cells attached to the mineral surface or free-swimming (planktonic phase) cells. FOB, iron-oxidizing bacteria; SOB, sulfur-oxidizing bacteria.

is the same, though the proximity of the microbe to the mineral surface may differ.

The notion that attached *At. ferrooxidans* cells can accelerate the oxidative dissolution of acid-soluble and -insoluble sulfides over and above that of ferric iron attack on the minerals has been challenged (Fowler and Crundwell, 1998; Fowler *et al.,* 1999). Using an apparatus in which the redox potential ($[Fe^{2+}]/[Fe^{3+}]$ ratios) could be accurately maintained, they showed that the presence of bacteria had no effect on zinc sulfide dissolution when a high redox potential was maintained abiotically, and concluded that the sole role of the bacteria was the regeneration of ferric iron in solution. In the case of pyrite, however, the rate of pyrite oxidation was greater in the presence than in the absence of *At. ferrooxidans* when the same redox was maintained. Fowler *et al.* (1999) concluded that this was as a result of the attached bacteria increasing the pH at the surface of the pyrite, thereby causing enhanced dissolution.

IV. Ecology, Applications, and Environmental Significance

A. Culturing Acidophilic Prokaryotes

Acidophilic bacteria and archaea may be successfully subcultured and maintained in appropriate liquid media. These media vary in composition according to the nutritional requirements of the organism(s) concerned. For example, "9K" is the much cited medium for growing iron-oxidizing autotrophic acidophiles (Silverman and Lundgren, 1959). However, 9K has the twin disadvantages of containing an excess of phosphate and a rather high (for an acidophilic medium) initial pH which, combined with the large concentration of ferrous present (9 g/liter) promotes the formation of large quantities of ferric iron rich precipitates in the form of jarosite and ferrihydrite. This, in turn, makes recovery of cells (which tend to stick to these precipitates) rather difficult. This problem can be eliminated by changing the composition of the liquid media to one containing a lower phosphate concentration and lower starting pH. As described in Section III.A.1, the oxidation of ferrous iron is a proton-consuming reaction, and it is necessary to lower the pH at which the ferrous iron medium is initially poised if large concentrations of ferrous iron (as substrate) are used in batch cultures. For example, a 9K-based formulation (equivalent ferrous iron concentration ~160 m*M*) requires an initial pH of 1.5–1.6 to eliminate the potential precipitation problem, and this might require that the cultures are first adapted to grow at such low pH.

On the other hand, sulfur-oxidizing acidophiles tend to lower the pH of their growth medium (due to the production of sulfuric acid) and the general practice is to start such cultures at pH > 2.0. Some sulfur oxidizers, notably *H. acidophilus* (Sec. II.A.2), may lower culture pH to the extent that they become either inactive or die (P. R. Norris, University of Warwick, personal communication). Both of these problems derive from the fact that liquid media used routinely for growing acidophiles are often poorly buffered, due to the lack of an appropriate (and nontoxic) buffering system at these low pH values. The most useful buffering system is, in fact, sulfuric acid itself, which has a $pK_{a2}(HSO_4^- + H^+ \leftrightarrow SO_4^{2-} + 2H^+)$ at pH 1.92. The problem of microbiological consumption or production of proton acidity can be eliminated by growing acidophiles in pH-controlled fermentor cultures.

Growth of acidophilic microorganisms on (agar-gelled) solid media has often been found to be difficult, with either very low plating efficiencies or no colony growth at all. Using a dual-layer approach, in which the lower layer of an agarose-gelled plate contains viable heterotroph, usually *Acidiphilium* strain SJH, it has been found possible to grow, with high plating efficiencies, all known species of mesophilic and moderately thermophilic iron- and sulfur-oxidizing acidophiles (Johnson, 1995). Solid medium formulation may be subjected to variation [e.g., type of (inorganic) substrates incorporated, pH] in the basic design of this "overlay" technique, to select for different physiological groups of acidophilic bacteria and archaea. Media of this type have been used successfully to assess the acidophilic biodiversity of environmental and commercial mineral leaching samples.

A major problem facing commercial biooxidation operations is the initial startup of these expensive plants. The need for rapid growth of extremely large acidophilic populations in a relatively short period of time is important for successful commissioning of such an operation. Therefore, dependable methods of growth of mixed populations of acidophiles is of importance. One method proposed (Pronk *et al.,* 1991b) makes use of the relatively fast growth rate and relatively high growth yield during growth on formic acid to obtain a large amount of *At. ferrooxidans*. Similar approaches may well work with other acidophiles that are of more relevance to a biooxidation operation. Another problem facing commercial operators is the preservation of acidophiles on a large scale. Several approaches for preserving acidophiles work well in the laboratory and include freezing at −70°C using glycerol or dimethyl sulfoxide (DMSO) as cryoprotectant. For a large-scale operation, however, it would be impossible to ship cultures to be used as inoculum in such a preserved manner. Methodologies for freeze-drying acidophiles on a large scale need to be addressed.

B. Methods for Enumerating and Identifying Acidophiles

Several approaches have been devised for enumerating and identifying acidophilic bacteria and archaea, which may be delineated as classical, immunological, and nucleic acid based methodologies (Jerez, 1997).

1. *Classical Techniques*

Most probable number microbial counts in specified liquid media (e.g., acidic ferrous sulfate medium) and incubated at an appropriate temperature has been used, and continues to be used, to enumerate different acidophiles on a physiological basis. This approach, however, suffers from drawbacks in that it does not distinguish between species that have similar metabolic capacities (e.g., *At. ferrooxidans* and *L. ferrooxidans,* both of which oxidize ferrous iron at 30°C in organic-free media). Plating on overlaid solid media (Sec. V.A) can give accurate assessments of sizes of acidophilic microbial populations, and variations in colony morphologies may be used as preliminary guidelines for identifying isolated acidophiles (Johnson and Roberto, 1997). This approach has also been the basis for isolating previously unknown acidophiles from environmental samples (Johnson, 2001).

2. *Immunological Techniques*

The classical approaches described above can yield very useful information concerning population sizes and diversity. They suffer, however, from several drawbacks, not the least of which is the lengthy time needed for incubation of microorganisms before results can be obtained. Therefore, more rapid approaches for enumerating and identifying microorganisms are needed if they are to be used in an industrial setting such as bioleaching tanks.

To this end, immunoassays have been adopted to enumerate acidophiles. In these assays, acidophiles are applied to nitrocellulose membranes (usually by vacuum) and the acidophiles are then detected following addition of antibodies that cross-react with components on the acidophiles. Specific antibodies that differentiate various species of acidophiles include those specific for *At. ferrooxidans* (Apel *et al.,* 1976; Arredondo and Jerez, 1989; Muyzer *et al.,* 1987), *L. ferrooxidans* (Jerez and Arredondo, 1991), and *At. caldus* and *Sulfolobus* (probably *S. metallicus*) strain BC65 (Amaro *et al.,* 1994). While this approach can be very strain specific and sensitive (able to detect about 10^3 microbes), the existence of multiple serotypes among Gram-negative acidophiles (Hallberg and Lindström, 1996; Koppe and Harms, 1994) indicates that immunoassays may be of limited use. A thorough knowledge of the microbes present in a leaching operation and a judicious choice

of antibodies, which target cellular components other than the variable LPS molecules that give rise to serotypes, could easily help overcome the problems of serotypes.

Another problem with immunoassays in a leaching setting is the enumeration of microorganisms that are attached to metal sulfides. Early attempts to overcome this problem include the addition of various detergents to remove attached microbes followed by centrifugation to separate both the planktonic and the recently detached microbes (Muyzer *et al.,* 1987). Studies with bacteria that were labeled with a radioisotope (such as ^{14}C) showed that such treatment can result in recovery of 100% of the bacteria from mineral surfaces (Garcia and Jerez, 1995). An innovative solution to this problem is the recently described enzyme-linked immunofiltration assay (ELIFA) (Dziurla *et al.,* 1998). ELIFA uses specially adapted 96-well plates that have filters in the wells, which retain both planktonic microorganisms and those attached to mineral particles. Following a washing step, the bacteria can be detected with antibodies that have been cross-linked with an enzyme, which usually produces a colored product or a fluorescent product. Providing care is taken, ELIFA is reported to be able to allow detection of all microbes on the mineral particles that were applied to the wells.

A further use of an immunoassay is to monitor the physiological state of the microbes. Following study of the response of *At. ferrooxidans* to phosphate starvation, antibodies specific to an extracellular protein that is expressed under phosphate-starvation conditions were generated (Varela *et al.,* 1998). Such an assay could provide a very quick picture of the health of the microbial population in mineral processing bioreactors long before a problem could lead to the collapse of the population and the resulting operation.

3. *Nucleic Acid Based Techniques*

16S rRNA gene technology has also transformed the study of microbial ecology, allowing the study of microbial communities without prior cultivation (Hugenholtz *et al.,* 1998). The most commonly used techniques in molecular microbial ecology have been described in a number of review articles (Amann *et al.,* 1995; Head *et al.,* 1998). In general, the biodiversity of microbes in a particular ecological niche can be assessed by extraction of nucleic acid from that environment followed by amplification of the 16S rRNA gene (16S rDNA) by polymerase chain reaction (PCR) using universal primers or strain specific primers. The PCR products can then be cloned and the resulting gene library may be screened using a variety of techniques. The 16S rRNA genes can be sequenced and the sequences compared with previously published data to assess the biodiversity of the sample, and where possible, to identify individual clones.

In the mid-1990s the first reports appeared in the literature describing the application of molecular-based methodologies to acidophiles that are important to biomining, though the number of such publications remains relatively sparse. In the first such studies, 16S rRNA genes from 33 representative strains of acidophilic bacteria, obtained from enrichment cultures using acidic runoff from a chalcocite overburden heap and leachate liquors from laboratory-scale bioreactors as inocula, were cloned (Goebel and Stackebrandt, 1994a, 1994b). Gene sequence data indicated that all of the clones from the environmental sample and the bioreactor run in batch mode consisted of a variety of iron- and sulfur-oxidizing bacteria and heterotrophic acidophiles, but the biodiversity in the bioreactor run in continuous mode was more limited. The microbes enriched from this reactor consisted of a *L. ferrooxidans*-like strain, a moderately thermophilic iron oxidizer belonging to the genus *Sulfobacillus,* and a moderately thermophilic sulfur oxidizer, later confirmed as *At. caldus*. The true biodiversity in this reactor, however, was not directly assessed.

To avoid introducing bias due to the enrichment step, the same researchers obtained 120 clones using DNA prepared from acidic (pH 1.5) run-off from a chalcocite overburden mound (Goebel and Stackebrandt, 1995), and again found that all of these clones were closely related to previously cultured acidophiles. More recently, 16S rRNA-gene libraries have been prepared from DNA samples obtained at an abandoned pyrite mine at the Iron Mountain site (Bond *et al.,* 2000b) and acidic geothermal sites on the volcanic island Montserrat (Burton and Norris, 2000). In the latter studies, a greater diversity of microorganisms was found, probably reflecting the differences in the environments surveyed (dynamic environmental systems as opposed to the relatively stable bioreactors).

Biomolecular approaches other than cloning and sequencing have also been used for the rapid analyses of mineral-leaching populations. These include restriction enzyme mapping of the 16S rDNA amplified from bioleachate liquors (Rawlings, 1995; Rawlings *et al.,* 1999a), often referred to as "ARDREA" (*a*mplified *r*ibosomal *D*NA *r*estriction *e*nzyme *a*nalysis). A second method for the rapid analysis includes the design and use of microbe-specific PCR primers based on known sequence data (De Wulf-Durand *et al.,* 1997). Here, selected primers based on published 16S rRNA sequences for three species and three genera of acidophilic bacteria were designed and used to amplify the 16S rRNA genes using DNA extracted from the bioreactor as template. Use of nested PCR, that is PCR using univeral 16S rRNA gene primers followed by a second PCR with the specific primers, resulted in a sensitivity level such that, theoretically, as few as 50 cells of the target organism in the original sample could be detected.

Other genetic markers that can be amplified with "universal PCR primers" and used to rapidly discriminate between acidophiles include the spacer region between the 16S and 23S rRNA genes of acidophiles, which can be discriminated based on size (Pizarro *et al.,* 1996). Partial sequencing of the 16S rRNA genes adjacent to the spacer regions of various sizes that are obtained can be sequenced to confirm which organism is represented by a specific amplified spacer region.

Although the 16S rRNA gene is the most common target in PCR-based studies of acidophilic microorganisms, other genetic material can also be targeted. For example, denaturing gradient gel electrophoresis (DGGE) was used to separate 5S rRNA which had been extracted from acidophilic biomass (Stoner *et al.,* 1996). Migration patterns of 5S rRNA from different acidophilic bacteria were readily distinguishable from each other, and this allowed the species composition of a mixed microbial community to be readily assessed. While DGGE is a very discriminating technique for the study of nucleic acids from microbial populations (Muyzer, 1999), this approach suffers from the need to have sufficient biomass to be able to detect the genetic material studied.

The PCR-based technique involving the random amplification of polymorphic DNA (RAPD) was applied to the study of *At. ferrooxidans* (Novo *et al.,* 1996), where two different groups of strains were identified (see discussion on phylogeny of *At. ferrooxidans* in Sec. II.A.4.C). In RAPD, a variety of oligonucleotides are synthesized and used for the amplification of short segments of DNA. Since the oligonucleotides are designed to bind to DNA randomly during the annealing step of PCR, no prior DNA sequence information from microorganisms is needed. In another study, the discrimination of thiobacilli using RAPD was accompanied by the use of two other PCR-based techniques, namely ARDREA and rep-APD (Selenska-Pobell *et al.,* 1998). Rep-APD makes use of primers that are specific to short, repetitive DNA segments, which are found in most microorganisms, to amplify the DNA between these repeats. The segments of DNA that are amplified vary from one strain to another and are thus a useful method for quick comparative genome analysis. The authors of this study found that both RAPD and rep-APD were much more discriminatory than ARDREA, where limited genetic material is available for comparison.

Although the approaches described above are useful in assessing environmental biodiversity, they are not quantitative. They all rely on the use of PCR for the amplification of the target gene, a process which has been shown to be subjected to various biases (von Wintzingerode *et al.,* 1997). Enumeration of different microorganisms, including those which have not actually been isolated and cultured, may be achieved using fluorescently labeled oligonucleotide probes ("genomic paints")

that may be designed to various levels of specificity (Amann *et al.,* 1990) in a process called fluorescent *in situ* hybridization (FISH). By comparing the number of microorganisms in a population that hybridize to a particular probe to the total number of cells present (obtained using a general fluorescent stain such as the DNA-binding stain 4′,6-diamidino-2-phenylindole or DAPI), it is possible to assess a particular microbe's numerical abundance.

Using rRNA probes based on previously reported sequences (Table III), the diversity of microbes present in slimes and water in an abandoned mine at Iron Mountain, California, was assessed (Bond *et al.,* 2000a; Edwards *et al.,* 1999; Schrenk *et al.,* 1998). Once again, a large diversity of microorganisms was observed. FISH has also been applied to laboratory reactors and environmental samples (Peccia *et al.,* 2000) using a probe developed to identify members of the genus *Acidiphilium* and one to identify *At. thiooxidans* and *At. ferrooxidans* (Table III). For rapid counting of numbers of microbes in a mixed population, fluorescent probes can be combined with flow cytometry (Porter and Pickup, 2000), a technique that may be more suited to industrial processes such as biomining.

C. Microbial Ecology of Extremely Acidic Environments

As described in earlier sections, acidophiles span a range of microorganisms, many of which have similar or complimentary physiologies. As with more "normal" environments, interactions between acidophilic microorganisms are many and various (Table IV); this topic has been the subject of recent reviews (Johnson, 1998a, 2001). A major difference between many extremely acidic and "normal" environments is the central importance of chemoautotrophs (iron and sulfur oxidizers) in primary production in the former, though photosynthetic acidophiles (e.g., the moderately thermophilic rhodophyte *Cyanidium caldarium* and the eukaryote *Euglena mutabilis*) may contribute substantially to net CO_2 fixation in illuminated acidic environments.

The end products of oxidative chemolithotrophy (ferric iron and sulfate) may serve as terminal electron acceptors for other microflora in neighboring anoxic environments (e.g., minerals tailings sediments) which, in turn, may generate electron donors for the former. Mutualistic interactions of this type may involve acidophiles of a single species located in juxtaposed environments. For example, ferric iron generated by *At. ferrooxidans* in aerobic zones may diffuse to neighboring anoxic zones and act as a terminal electron acceptor for cells of the same species respiring anaerobically on elemental sulfur and RISCs which, in turn, regenerates ferrous iron. The net effect is the net oxidation of sulfur and

TABLE III

DOMAIN-SPECIFIC OR ACIDOPHILE-SPECIFIC OLIGONUCLEOTIDE PROBES THAT TARGET THE 16S rRNA MOLECULE

Probe name	Target organism	Sequence (5′-3′)	Reference
UNIV1392	Universal (all organisms)	ACGGGCGGTGTGTRC	Olsen *et al.,* 1986
EUK502	Eukaryotes	ACCAGACTTGCCCTCC	Amann *et al.,* 1990
EUB338	Eubacteria	GCTGCCTCCCGTAGGAGT	Ibid.
ARCH915	Archaea	GTGCTCCCCCGCCAATTCCT	Stahl and Amann, 1991
NON338	No target[a]	ACTCCTACGGGAGGCAGC	Wallner *et al.,* 1993
TH1187	*Thermoplasmales*	GTACTGACCTGCCGTCGAC	Bond and Banfield, 2001
FER656	*Ferroplasma*	CGTTTAACCTCACCCGATC	Edwards *et al.,* 2000b
F581	*Leptospirillum* groups I and II[b]	CGGCCTTTCACCAAAGAC	Schrenk *et al.,* 1998
F1252	*Leptospirillum* group III[b]	TTACGGGCTCGCCTCCGT	Bond and Banfield, 2001
F655	*Leptospirillum* groups I, II and III[b]	CGCTTCCCTCTCCCAGCCT	Ibid.
ACM732	*Acidimicrobium* and relatives[c]	GTACCGGCCCAGATCGCTG	Ibid.
SUL228	*Sulfobacillus thermosulfidooxidans*[d]	TAATGGGCCGCGAGCTCCC	Ibid.
ACD840	*Acidiphilium* genus	CGACACTGAAGTGCTAAGC	Ibid.
Acdp821	*Acidiphilium* genus	AGCACCCCAACATCCAGCACACAT	Peccia *et al.,* 2000
Thio820	*Acidithiobacillus* spp[e]	ACCAAACATCTAGTATTCATCG	Ibid.
TF539	*At. ferrooxidans*	CAGACCTAACGTACCGCC	Bond and Banfield, 2001
THC642	*At. caldus*	CATACTCCAGTCAGCCCGT	Edwards *et al.,* 2000a

[a] This probe has no target in the 16S rRNA. It is used as a control to check nonspecific probe binding.

[b] The *Leptospirillum* grouping is based on phylogenetic analysis of environmental clones from the Iron Mountain site (Bond *et al.,* 2000b).

[c] This probe targets *Ac. ferrooxidans* as well as related clones from Iron Mountain and "*Ferrimicrobium acidiphilum*," but not the environmental clones IMBA84 and TRA2-10 from Iron Mountain.

[d] This probe also targets *Sb. acidophilus*, but with a one-base mismatch in the target region, and thus it may not be specific for *Sb. thermosulfidooxidans* under certain hybridization conditions.

[e] *At. thiooxidans*, *At. ferrooxidans*, and *Acidithiobacillus* sp. DSM612 (previously known as *T. thiooxidans* DSM612) are the targets for this probe.

TABLE IV

EXAMPLES OF INTERACTIONS BETWEEN ACIDOPHILIC MICROORGANISMS

Interaction	Description	Example
Competition	Populations of two or more species are mutually limited due to joint dependence on a common substrate	*At. ferrooxidans*/*At. thiooxidans* (S^o, RISCs); *At. ferrooxidans*/*L. ferrooxidans* (Fe^{2+})
Mutualism sp.	Association of two organisms from which both benefit	*L. ferrooxidans*/*Acidiphilium* (organic C interactions); *At. caldus/Sb. thermosulfidooxidans* (pyrite leaching)
Synergism	Association of two (or more) organisms with complimentary activities allowing them to grow, when together but not in pure culture	*At. thiooxidans* or *A. acidophilum*/"*Fm. acidiphilum*" (pyrite leaching)
Ammensalism	Repression of one species by toxins produced by another	*At. thiooxidans*/*Acidocella* spp. (H^+ production from S^0/RISC oxidation)
Predation	Ingestion of one organism by another	Acidophilic flagellates and ciliates grazing acidophilic bacteria

RISCs and reduction of molecular oxygen, with iron acting as a redox shuttle. Temperature constraints on acidophiles will have an important bearing on microbial ecology and interactions, e.g., in determining which bacteria and/or archaea are competing for ferrous iron (as electron donor) in space and time.

D. BIOPROCESSING OF SULFIDIC ORES

The use of acidophilic microorganisms to solubilize sulfide minerals, and thereby facilitate extraction and recovery of metals, is a technology that has its roots long before the era of modern microbiology. "Precipitation ponds," within which sulfide-rich rocks and boulders were subjected to leaching, were in place in the 18th and 19th centuries at Rio Tinto (Spain) and Parys Mountain (Wales), both of which are copper mining sites which date back to pre-Roman times. "Biomining" as a recognized "novel" technology took hold in the 1960s, following the characterization of *At. ferrooxidans,* and demonstration that it could be used to accelerate the dissolution of metal sulfides (Colmer *et al.,* 1950). The earliest engineering technology used ("dump leaching") was very basic, involving gathering low-grade (otherwise waste) copper-containing ore

of large rock/boulder size into vast mounds or dumps and irrigating these with dilute sulfuric acid to encourage the growth and activities of mineral-oxidizing acidophiles, primarily iron-oxidizing mesophiles. Copper was precipitated from the metal-rich streams draining from the dumps by displacement with scrap iron ("copper cementation").

Later developments on the engineering and hydrometallurgical aspects of biomining have involved the use of thin layer heaps of refractory sulfidic ores (mostly copper, but also gold-bearing material) stacked onto water-proof membranes, and solubilized copper recovered using solvent extraction coupled with electrowinning (SX/EW). *In situ* bioleaching was developed to scavenge for uranium and copper in otherwise worked-out mines. This involves fracturing underground workings using explosives, percolating with acidic leach liquors containing metal-mobilizing bacteria, pumping the pregnant liquor to the surface, and extraction of solubilized metals. Most recently, aerated stirred tanks have been used to process sulfidic ore concentrates. These tanks, which may be extremely large (up to 1350 m^3), allow for greater control (e.g., of temperature; sulfide mineral oxidation being an exothermic process) of biooxidation of mineral ores. To date, stirred tank bioreactors used for mineral processing have tended to operate between 40 and 50°C (i.e., where moderate thermophiles and thermotolerant acidophiles would tend to be of greatest significance). However, it is anticipated that bioreactors operating at higher temperatures (>70°C), where thermophilic archaea would be predicted to dominate the active microflora, will be required to bioleach chalcopyrite concentrates (Norris *et al.,* 2000).

An excellent review of "biomining" has been written by Brierley (1997), which provides much of the information in Table V. This is part of a recent text (Rawlings, 1997) which gives comprehensive coverage of biological ore processing. More condensed reviews of the subject are also available (e.g., Johnson, 1995; Olson, 1994; Rawlings and Silver, 1995).

E. Acid Mine Drainage

The genesis of acid mine drainage, a cause of severe environmental pollution in those parts of the world with current or historic coal or metal mining industries is, in many ways, the flip-side of the biomining coin. Mineral-oxidizing prokaryotes are ubiquitous in sulfidic ores and coals. For example, iron-oxidizing acidophiles have been isolated from a pyritic coal sample taken from a freshly cut seam at an open-cast site (Johnson, unpublished data). On exposure to both air and water, pyrite and other sulfide minerals are subject to oxidative dissolution [producing, ultimately, ferric iron, sulfate, and protons;

TABLE V

SOME MILESTONES IN THE DEVELOPMENT OF BIOPROCESSING OF MINERAL ORES USING ACIDOPHILIC PROKARYOTES ("BIOMINING")

Date	Location	Operation
18th and 19th centuries	Spain, UK	Leaching of copper ores in precipitation ponds
1960s	USA	Copper dump leaching (Kennecott Corporation)
late 1960s–early 1990s	Ontario, Canada	*In situ* mining of uranium
1980–1996	Lo Aguirre, Chile	Bioheap leaching of copper (with SX/EW)
1993–	Girilambone, New South Wales, Australia	Bioheap leaching of copper (with SX/EW)
1995–	Nevada, USA	Bioheap leaching of gold ore
1986–	Fairview, South Africa	Bioprocessing of gold ore concentrate in aerated stirred tanks
1990–	Sao Bento, Brazil	Single stage stirred tank (gold ore concentrate)
1994–	Sansu, Ghana	Bioprocessing of gold ore concentrate in aerated stirred tanks
1999–	Kasese, Uganda	Bioprocessing of cobaltiferous ores in stirred tank bioreactors

Eqs. (4)–(7)]. Even in anoxic zones within mines and mine spoils, sulfides are susceptible to oxidative dissolution, mediated by ferric iron and resulting in the production of ferrous iron, protons, and sulfur compounds of varying oxidation states. For the latter process to be ongoing, a continuous influx of soluble ferric iron is required. This will occur, for example, in a mineral spoil heap where an outer, aerated zones shrouds an inner (and generally more voluminous) anoxic core.

The acidic, metal-rich wastewaters which flow from abandoned mines, spoil heaps, and tailings are generally referred to as acid mine (or rock) drainage (AMD/ARD). The composition of AMD/ARD is highly variable (e.g., Banks *et al.,* 1997; Johnson *et al.,* 2001c), both in terms of pH, acidity, and concentrations of dissolved solids. Total acidity in AMD waters is a summation of proton acidity (i.e., pH) and mineral acidity, the latter deriving from the presence of iron (ferrous and ferric), aluminum, and manganese, each of which can give rise to proton

genesis on hydrolysis:

$$Al^{3+} + 3H_2O \rightarrow Al(OH)_3 + 3H^+. \quad (8)$$

The presence of soluble aluminium (deriving mostly from acid dissolution of aluminosilicate minerals) is one of the main reasons why AMD is highly toxic to most life forms. Incidents involving catastrophic release of AMD into water courses (e.g., Aznalcollar, Spain 1996; Fal estuary, Cornwall, UK, 1991) has resulted in massive fish kills. Remediation of AMD has traditionally involved chemical treatment (liming to raise pH and aeration to oxidize ferrous iron), but more recently biological treatment (in constructed wetlands, or using sulfidogenic bioreactors as a source of hydrogen sulfide to remove chalcophilic metals) has gained in popularity (Johnson, 2000).

F. Other Applications of Acidophiles in Biotechnology

Thus far, acidophilic microorganisms have been exploited commercially only in biomining operations. There are other situations in which acidophilic bacteria and archaea may, in theory, be of potential use, including bioremediation of AMD. The use of immobilized populations of *At. ferrooxidans* to oxidize ferrous iron in acidic wastewaters, which (at pH >2.5) will result in the hydrolysis and precipitation of the ferric iron produced, has been considered as an alternative to, or used in conjunction with, chemical treatment (e.g., Gomez *et al.,* 2000; Nemati and Webb, 1999). Iron-reducing acidophiles (e.g., in fixed bed reactors) have been demonstrated to be capable of rapid and efficient reduction of ferric iron to ferrous, as part of a novel two-phase approach for bioremediating extremely acidic mine wastewaters (Johnson *et al.,* 2000). Acid-tolerant (growing at pH 3 and above) sulfate-reducing bacteria have been isolated from "acid streamers" (macroscopic growths of microbes) and anoxic sediments (Johnson *et al.,* 1993; Sen and Johnson, 1999). These could conceivably be used to generate hydrogen sulfide (and alkalinity) in acidic wastewaters; currently, offline SRB reactors are required in order to shield acid-sensitive sulfidogenic bacteria from the toxic effects of AMD. Finally, there are as yet untapped areas of biotechnology (e.g., as sources of acid-, and possibly heat-, tolerant enzymes, and biodegradation of organic pollutants) in which acidophilic prokaryotes could have considerable potential.

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Laboratory Bioproduction of Paralytic Shellfish Toxins in Dinoflagellates

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I. Introduction

Paralytic shellfish toxins (PSTs) are a family of marine biotoxins produced by a number of toxic dinoflagellates involved in harmful algal blooms or red tides. They are also produced by cyanobacteria (Pomati *et al.,* 2000) in fresh waters. These toxins cause paralytic shellfish

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0065-2164/01 $35.00

poisoning (PSP) which is the most common and widespread syndrome among the major classes of seafood poisonings recognized today (Viviani, 1992; Hallegraeff, 1995).

A. Toxic Action and Chemistry of PSTs

PSTs consist of over 20 structurally related compounds that have been chemically characterized. The toxins act by binding to sodium channels of the nervous system and disturb the initiation of action potentials resulting in a blockage of neuromuscular transmissions and hence the name paralytic shellfish poisoning. Some symptoms of human intoxication include tingling sensations in the extremities, headaches, nausea, and vomiting. In extreme cases, PSP can lead to respiratory paralysis (Andrinolo *et al.,* 1999) and death if artificial respiration is not available. Based on toxic potency, they can be subdivided into three groups as shown in Table I. In terms of toxicity measured by mouse bioassay, the carbamate compounds, which include saxitoxin (STX), neo-saxitoxin (Neo), and gonyautoxins 1-4 (GTX1-4), are most toxic, the N-sulfocarbamoyl compounds, which include the C and B toxins, are least toxic (up to 100 times less), while the decarbamoyl compounds have intermediate toxicity. Saxitoxin, being the first PST to be identified, has been the most widely studied toxin derivative and is also the most toxic of the entire PST family (Oshima, 1995).

PSTs are heat-stable and water-soluble nonproteinaceous toxins. They are small molecular weight tetrahydropurines (242–491 g/mol) that have the same basic chemical structure differing only in their side groups. The general structures of these compounds are shown in Figure 1. These analogues differ with respect to the presence or absence of 1-N-hydroxyl, 11-hydroxysulfate, and 21-N-sulfocarbamoyl substitutions as well as epimerization at the C-11 position.

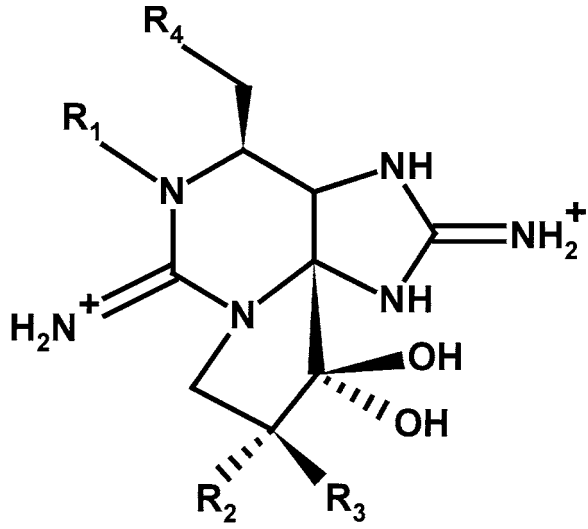
B. PST-Producing Marine Dinoflagellates

PSTs were originally isolated from contaminated shellfish but later found to originate from marine algae. These compounds are now known

TABLE I

Three Groups of PSTs Based on Toxic Potency

Chemical class	Potency	Toxins
Carbamate	High	Saxitoxin (STX), neo-saxitoxin (NEO), gonyautoxins (GTX)-1, -2, -3, -4
Decarbamoyl	Medium	dc-GTX 1-4, dc-NEO, dc-STX
N-Sulfocarbamoyl	Low	B1, B2, C1, C2, C3, C4,



Molecular Weight	R1	R2	R3	R4	Toxin
242.3	H	H	H	H	doSTX
258.3	H	H	H	H	dcSTX
274.3	OH	H	H	H	dcNEO
301.3	H	H	H	$CONH_2$	STX
317.3	OH	H	H	$CONH_2$	NEO
337.3	H	OSO_3^-	H	H	doGTX2
337.3	H	H	OSO_3^-	H	doGTX3
353.3	H	OSO_3^-	H	H	dcGTX2
353.3	H	H	OSO_3^-	H	dcGTX3
369.3	OH	OSO_3^-	H	H	dcGTX1
369.3	OH	H	OSO_3^-	H	dcGTX4
380.4	H	H	H	$CONHSO_3^-$	B1
396.4	OH	H	H	$CONHSO_3^-$	B2
396.4	H	OSO_3^-	H	$CONH_2$	GTX2
396.4	H	H	OSO_3^-	$CONH_2$	GTX3
412.4	OH	OSO_3^-	H	$CONH_2$	GTX1
412.4	OH	H	OSO_3^-	$CONH_2$	GTX4
475.4	H	OSO_3^-	H	$CONHSO_3^-$	C1
475.4	H	H	OSO_3^-	$CONHSO_3^-$	C2
491.4	OH	OSO_3^-	H	$CONHSO_3^-$	C3
491.4	OH	H	OSO_3^-	$CONHSO_3^-$	C4

STX:saxitoxin; NEO:neosaxitoxin; GTX:gonyautoxin; dc:decarbamoyl; do:deoxydecarbamoyl

FIG. 1. Chemical structures of PST.

to be produced by a limited number of species of unicellular marine algae known as dinoflagellates as listed in Table II. At present, only three genera of dinoflagellates are known to produce PSTs. Most of the species are of the genus *Alexandrium,* which was formerly known as either *Gonyaulax* or *Protogonyaulax* (Taylor, 1987). Of the *Alexandrium,* the most commonly cultured species to date have been *A. tamarense*

TABLE II

A COMPILATION OF CONDITIONS OF SOME ALGAL CULTURES USED IN THE LABORATORY PRODUCTION OF PSTs

Studies	1	2	3	4
Species and location	*A. tamarense* (Canada)	*A. catenella*	*A. tamarense* (Canada)	*A. tamarense* (Japan)
Innoculum	NG[e]	1×10^4 cells/ml or greater	Exponential, 10% of final volume	NG
Culture duration (days)	117	15	36	20
Media formula	ASP7	Complex	Harrison Enrichment (HESNW)	T1
Culture design	Batch	Batch	Batch	Batch
Culture volume	125, 250 ml	2 Liter	2 Liter	NG
Culture vessel	20×125 mm Culture tubes, 125- and 250-ml Erlenmeryers	Fernbachs	NG	NG
Temperature (°C)	5–25	12–16	18	8–16
Salinity (ppt)	7–40	NG	28	NG
Light period (h)	24	14 and 24	16	16
Light Intensity[b] (μmol/m^2 sec)	80	80	1–25	10–60
Nitrogen (μM)	588	NG	0–550	1000
Phosphate (μM)	65	NG	0–24	100
Toxin composition[c]	NG	**STX**	**B and C toxins,** GTX1–4, Neo, STX	**GTX1–4,** Neo
Peak cellular toxin (fmol/cell)	NG	8.3	80	100
Toxin yield (nmol/liter of culture)	NC[f]	120	400[d]	657[d]
Method of analysis	Mouse assay	Mouse assay	HPLC	HPLC
Reference	Prakash (1967)	Proctor *et al.* (1975)	Boyer *et al.* (1987)	Ogata *et al.* (1987)

TABLE II—*Continued*

5	6	7	8
A. tamarense[a] *A. catenella*[a]	*A. catenella, A. cohorticula, G catenatum* (Japan, Thailand)	*A. fundyense*[a] (USA)	*A. fundyense, A. minutum* (USA)
Mid exponential	NG	Exponential, 5% of final volume	NG
60	20	NG	19
f/2	T1	K medium	K medium
Batch	Batch	Semicontinous	Batch and semicontinuous
2 Liter	NG	1 Liter	17 Liter
Fernbachs	NG	1-Liter reactors	1 Liter reactors 20 Liter carboys
15	10–25	15	15
NG	NG	30	28–38
16	16	14	14, 24
57	20–140	100	100–125
880	1000	44 to 883	22–933
36	100	0.9–10	0.25–36
STX, Neo, GTX1–4, B and C toxins	NG	**B1,** C toxins, GTX1–4, Neo, STX, B2	NG
55	150	1000	585
400[d]	NC	NC	2000
HPLC	HPLC	HPLC	HPLC
Boczar *et al.* (1988)	Ogata *et al.* (1989)	Anderson *et al.* (1990a)	Anderson *et al.* (1990b)

TABLE II—*Continued*

Studies	9	10	11	12
Speice and location	*G. catenatum* (Australia, Japan, Spain)	*A. catenella*[a] A. tamgrense[a] (Japan)	*P. bahamense* (Malaysia)	*A. minutum* (Spain)
Innoculum	NG	Exponential, 1×10^6 cells	Exponential	NG
Culture duration (days)	21	35	28	27
Media formula	Gse	SW11m	ES-1	Modified K medium
Culture design	Batch	Batch	Batch	Batch
Culture volume	NG	2 Liter	25 ml	NG
Culture vessel	50-ml Erlenmeyers	3 Liter flasks	Culture tubes	5-Liter vessel
Temperature (°C)	12.5–25	15	20–40	15
Salinity (ppt)	20–35	NG	20–36	NG
Light period (h)	12	14	24	12
Light Intensity[b] (μmol/m^2 sec)	80	100	20–150	180–200
Nitrogen (μM)	NG	NG	NG	100–300
Phosphate (μM)	NG	NG	NG	5–20
Toxin composition[c]	**C toxins,** dcSTX, GTX2/3, B toxins	**C Toxins,** GTX1–4, STX, Neo, B1, B2	**B1, Neo,** STX, GTX6, dcSTX	GTX1–4
Peak cellular toxin (fmol/cell)	200	66	1200	40
Toxin yield (nmol/liter of culture)	NC	330[d]	1000	400
Method of analysis	HPLC	HPLC	HPLC	HPLC
Reference	Oshima *et al.,* (1993)	Kim *et al.* (1994)	Usup *et al.* (1994)	Flynn *et al.* *et al.*

TABLE II—*Continued*

13	14	15	16
A. tamarense (Canada)	*A. catenella*[a] (Japan)	*G. catenatum* (Spain)	*A. tamarense* (Japan)
NG	Late exponential	NG	NG
15	30	20	60
Harrison Enriched Artificial Seawater (ESAW)	SW11m	Modified K medium	T1 and SWII
Batch	Batch and semicontinuous	Batch and continuous	Batch and semicontinuous
2.2 liter	NG	1 Liter	NG
4-Liter Erlenmeyers	NG	2-Liter flasks	300-ml Erlenmeyers
18	20	18	15
NG	35	22–33	NG
14	14	12	16
160	80	180	60
50–100	NG	50–200	20–1000
22	70	0–20	100
C toxins, Neo, STX, GTX1–4	**B1, C toxins,** GTX1–4, Neo dcSTX	**C toxins,** dcSTX, DcGTX, GTX5	NG
284	26	125	170
NC	240[d]	125[d]	1000[d]
HPLC	HPLC	HPLC	HPLC
Levasseur *et al.* (1995)	Matsuda *et al.* (1996)	Flynn *et al.* (1996)	Ogata *et al.* (1996)

TABLE II—*Continued*

Studies	17	18	19
Species and location	*A. catenella* (Hong Kong)	*A. minutum* (New Zealand)	*A. tamarense* (Canada)
Innoculum	Mid exponential, 4–5 $\times$ 10^3 cells/ml	NG	4 $\times$ 10^3 cells/ml
Culture duration (days)	40	NG	19
Media formula	K medium	f/2	K medium
Culture design	Batch	Batch	Batch
Culture volume	100–250 ml	25 ml	10 Liter
Culture vessel	1-Liter bottles	NG	15-Liter carboys
Temperature (°C)	10–30	18	15
Salinity (ppt)	15–45	31	NG
Light period (h)	10–18	14	14
Light intensity[b] (μmol/m^2 sec)	120	250	140
Nitrogen (μM)	0–8500	880	60–880
Phosphate (μM)	0 to 400	36	30
Toxin composition[c]	C toxins, GTX1–4, Neo, B2	**Neo,** STX, GTX1–4	**C toxins,** STX, Neo, B1, GTX2-4, dcGTX3
Peak cellular toxin (fmol/cell)	16	27	420
Toxin yield (nmol/liter of culture)	32[d]	NC	3400
Method of analysis	HPLC	HPLC	HPLC
Reference	Siu *et al.* (1997)	Chang *et al.* (1997)	Macintyre *et al.* (1997)

TABLE II—*Continued*

20	21	22	23
A. tamarense (Canada)	*A. minutum* (France)	*A. fundyense* (USA)	*A. minutum* (Taiwan)
Late exponential, 5 ml of 8×10^3 cells/ml into 30 ml	2.73×10^6 cells/ml	NG	5×10^4 cells
14	36	45	40
K medium	f medium	Modified K medium	Modified K medium
Batch	Semicontinuous	Batch	Batch
35 ml	4 Liter	1.5 Liter	500 ml
50-ml Culture tubes	5-Liter polycarbonate bottles	2-Liter round-bottom flasks	NG
15	18	18	10–30
15–35	35	28	7.5–37
14	16	12	24
40–470	53	150	15–240
0–880	1.6–60	50–430	0–2000
10	0.38 to 3.8	0.5 to 28	0 to 1300
C toxins, STX, Neo, GTX1–4	GTX2/3, dcGTX2/3	**GTX2/3,** STX, C toxins	**GTX1–4**
800	9	NG	172
2100[d]	NC	NC	NC
HPLC	HPLC	HPLC	HPLC and mouse assay
Parkhill and Cembella (1999)	Bechemin *et al.* (1999)	John and Flynn (2000)	Hwang and Lu (2000)

[a]Axenic culture.
[b]Converted to common units of photon flux density.
[c]Predominant toxin in boldface.
[d]Estimated by calculation.
[e]NG = Not given.
[f]NC = Not calculable.

(formerly known as *excavatum* or *tamarensis*), *Alexandrium catenella*, *A. minutum*, and *A. fundyense*. *Alexandrium cohorticula* also produces PSTs while another species, *A. andersoni*, has just recently been shown to produce PSTs (Ciminiello *et al.,* 2000). Isolated from tropical waters, *Pyrodinium bahamense* var. *compressum* is another species that causes PSP episodes, particularly in Indo-Pacific and Central America coastal waters. Usup *et al.* (1994, 1996) have done some work on this species which only appears to be toxic with the chain-forming var. *compressum*. Another toxigenic species among athecate *gymnodinoid* dinoflagellates is *Gymnodinium catenatum*, which is also a chain-forming species (Oshima *et al.,* 1993; Flynn *et al.,* 1996). This species is the most fragile of all the PST producing dinoflagellates because it is an unarmoured species.

C. The Need for Pure Toxins

Due to the potent toxicity of PST, mitigation of PST problems has in recent years received increasing regulatory attention worldwide. To this end, an ample supply of PSTs, in highly purified form, is urgently needed for use in clinical diagnostic laboratories, safety surveillance of seafood, development of fast monitoring methods, generation of toxicity data for risk assessment, and pharmacological research to design antidotes and remedies. In addition, recent studies have revealed that PSTs may have some clinical uses. For example, Pan (1998) has shown that PSTs are highly effective compounds for relieving withdrawal symptoms in opiate-addicted patients. However, at present there is a globally limited supply of pure marine biotoxins. Some toxins, available only as analytical standards, are either prohibitively expensive or simply unavailable. For example, while saxitoxin and neo-saxitoxin are available from Sigma and Calbiochem, GTX1-4 is only available from the Institute for Marine Biosciences, National Research Council, Canada. The cost of these PST derivatives can be as high as US$5000 per milligram. Other derivatives such as the C toxins and the decarbamoyl derivatives are simply not commercially available at the present time.

D. Bioproduction of Marine Biotoxins

Obviously, there is a very strong incentive to produce and purify these marine biotoxins. In general, two approaches can be taken to obtain pure biotoxins: one is bioproduction by cultured toxin producing algae and the other is extraction from toxin-bearing seafood. Once toxins are obtained from either algal or seafood extracts, they can be subject to

similar purification, analysis, and confirmation steps to meet the purity criteria for analytical standards and research compounds.

We have undertaken the bioproduction approach to attempt to produce milligram quantities of PST under laboratory conditions. In this article, we summarize the related works done by other investigators and describe our own work on the laboratory production of C2 toxin by a local strain of *Alexandrium tamarense*. We also discuss some of the conditions that appear to be important in influencing the toxin productivity of PST producing dinoflagellates.

II. Culture Designs and Physiology

The toxin productivity of a dinoflagellate culture is a function of the cell concentration and the average toxin content of each individual cell in the population. The total toxin yield of a bioproduction operation therefore depends on how well the cells grow and how much toxin each cell produces within a given period of time. The rate at which cells grow and produce toxins, in turn, is profoundly influenced by the culture design and the nutritional and environmental factors of the algal cultures used. Some known important factors include the supply of carbon, nitrogen, and phosphorus sources in the medium, the concentrations of metals and organic compounds present, irradiation, temperature, salinity, and mixing turbulence. The effects of some of these factors on the physiology and metabolism of dinoflagellates with respect to PST production were recently reviewed in detail by Cembella (1998). Our discussion on these factors is only related to operational aspects regarding the laboratory bioproduction of PSTs.

A. Culture Designs

In general, three designs of algal cultures can be used in the laboratory bioproduction of PST. They are batch, semicontinuous, and continuous culture designs. The designs that have been used for PST bioproduction studies and their associated culture conditions are compiled in Table II.

Of the three culture designs, batch cultures are the most commonly used. In batch cultures, algae are grown in vessels of different shapes such as tubules, bottles, flasks, and tanks with various nutrient media and culture conditions. The culture volumes range from several milliliters to tens of liters. Aeration is seldom used because it is believed that PST-producing dinoflagellates are sensitive to turbulence. It is generally understood that batch cultures begin as nutrient-unbalanced systems, in which algae are exposed to relatively high nutrient concentrations (e.g., $>500\ \mu M$ inorganic N and $>5\ \mu M$ inorganic phosphate) in

the culture medium. However, at some point during the batch culture, one or more nutrients will be depleted and become limiting. Some investigators observed that toxin productivity of certain dinoflagellates in batch cultures was lower than that under natural conditions (Kodama *et al.,* 1982; Maranda *et al.,* 1985; White, 1986; Oshima *et al.,* 1987; Cembella *et al.,* 1988). This may be due to the use of suboptimal culture conditions in the laboratory.

Semicontinuous cultures have been used in a few studies (Anderson *et al.,* 1990a; Bechemin *et al.,* 1999). In this design, a volume of aged growth medium is replaced with an equal volume of fresh medium at regular time intervals before the cell density reached a maximum. The dilution rate is guided by the cell growth rate. Semicontinuous cultures provide a relatively steady state condition for algal growth and toxin production.

Continuous cultures of microorganisms have been frequently used to fix the cellular growth rate by setting the dilution rate of the inflow medium that contains a limiting nutrient, thus eliminating the growth rate effect on a process of interest (Parkhill and Cembella, 1999). However, continuous cultures have seldom been used for the production of PSTs because most of the dinoflagellates are susceptible to growth inhibition by mixing turbulence. The only study known using this design is that of Flynn *et al.* (1996), who investigated the effects of inorganic nutrients and salinity on toxin production by *Gymnodinium catenatum*. In their study, although fresh medium was introduced continuously, excess medium was only removed once or twice a day.

Recently we have employed a combination of a semicontinuous culture and a holding or temporary culture to achieve mass production of C2 toxin by *Alexandrium tamarense*. This design capitalizes on the characteristic growth and toxin production dynamics of the production strain used to achieve very high yields of the toxin. The process is summarized in Section V, and the detailed process is described elsewhere (Wang *et al.,* 2001).

B. Algal Physiology

In batch cultures, algae normally go through three phases: the lag, the exponential, and the stationary phases. The average toxin content of cells has often been found to reach a peak in the exponential phase and then decline as the cells enter the stationary phase, with the exception that cessation of cell growth is caused by P limitation (Prakash, 1967; Proctor *et al.,* 1975; Hall, 1982; Boyer *et al.,* 1987; Cembella *et al.,* 1987; White and Maranda, 1978; Boczar *et al.,* 1988; Anderson *et al.,* 1990b; Kim *et al.,* 1993; Usup *et al.,* 1994; Flynn *et al.,* 1994, 1995, 1996;

Bechemin *et al.,* 1999; John and Flynn, 2000). But the exact relationship between growth rate and cellular toxin content is unclear. Some investigators found no definitive relationship between the two (White and Maranda, 1978; Hall, 1982; Parkhill and Cembella, 1999), whereas others observed an inverse relationship (Proctor *et al.,* 1975; Ogata *et al.,* 1987).

PSTs have been viewed as secondary metabolites of toxin-producing dinoflagellates, which are compounds produced when normal, balanced growth ceases (Plumley, 1997). Cells usually synthesize low amounts of secondary metabolites when growing under unstressed conditions but drastically increase the synthesis under certain conditions that limit growth (Hashimoto and Yamada, 1994; Jensen and Fenical, 1994). Our own data on *A. tamarense* were in part consistent with this view (Wang and Hsieh, 2001). During the exponential phase when cells grew freely, chl-a, a primary metabolite, was formed rapidly by the cells with little toxin production. But when algal growth ceased in the stationary phase due to nutrient depletion, chl-a disappeared in proportion to the increase of C2T.

There are reports indicating that PST production is tightly coupled to particular stages of the cell cycle. Kim *et al.* (1993) reported that cellular toxin content increased gradually from the later half of the light period through the middle of the dark period, where it declined suddenly prior to cell division. For *Alexandrium fundyense*, toxin synthesis was initiated early in the G1 phase of the cell cycle in response to a light trigger and was terminated prior to exiting this phase (Taroncher-Oldenburg *et al.,* 1999).

III. Nutritional Factors

For the bioproduction of PST by cultured algae, sufficient nutrients need to be supplied for the production of cell mass followed by toxin synthesis. Because cell growth and toxin production can be two competing processes for carbon, nitrogen, phosphoros, and other essential elemental supplies in the medium, the initial composition of nutrients must be optimized for the maximum total toxin yield in a batch culture. Supplementation of specific nutrients in the course of incubation can be achieved by the use of semicontinuous culture or other culture designs.

A. Carbon

Carbon is an essential element for cell growth, reproduction, and toxin biosynthesis. For toxigenic dinoflagellates, most being photoautotrophs, carbon is supplied by atmospheric CO_2 through photosynthetic fixation.

Carbon usage in the algal cells is normally first channeled to the primary metabolism related to growth and reproduction, and then to the secondary metabolism related to PST synthesis. If the available carbon is limited, toxin production will be reduced or even arrested in favor of primary metabolism. Therefore, in order to maximize PST production, dinoflagellate cultures should be given carbon in excess to ensure that neither growth nor toxin synthesis is retarded. Usually, CO_2 is not limiting due to the rapid dissolution of CO_2 into seawater to form aqueous bicarbonate. However, for laboratory cultures, when biomass is high, CO_2 consumption by the culture may exceed CO_2 dissolution into the medium due to the limitation of culture designs, causing the culture to be carbon limited. Boyer *et al.* (1987) and Anderson *et al.* (1990b) both found that addition of bicarbonate to cultures of *A. tamarense* enhanced growth. In our laboratory, we also found supplementation of bicarbonate as well as aeration (discussed in Section IV.D) increased both cell biomass and toxin yield.

B. Nitrogen

PST are a suite of nitrogen-rich compounds that require a sufficient supply of intracellular nitrogen-containing intermediates such as amino acids for their biosynthesis. With six nitrogen atoms per molecule of PST, nitrogen can account for 17–35% of the weight of a PST molecule. In addition, it was estimated that PSTs may account for up to 10% of the total cellular nitrogen in *Alexandrium* and *Gymnodinium* sp. (Cembella, 1998). As expected, nitrogen deficiency caused dinoflagellate cultures to decrease in PST yield and cellular toxin content (Anderson *et al.,* 1990a; Flynn *et al.,* 1994; MacIntyre *et al.,* 1997; John and Flynn, 2000). This decrease was readily reverted by nitrogen supplementation. Cellular toxin content typically peaked in the exponential phase when nitrogen supply was sufficient but markedly decreased in the stationary phase when nitrogen had become limiting. In our lab, we found that by reducing the nitrogen in the culture medium, cell density could still be maintained but the toxin content was markedly lowered. Hence, the supply of ample nitrogen appears to be important for maximum toxin yield.

C. Phosphorus

Although PST molecules do not contain phosphorus, the supply of phosphorus in the medium profoundly affects PST biosynthesis. Enhancement of bioproduction of PSTs by phosphorus limiting culture conditions has been well documented (Boyer *et al.,* 1987; Anderson *et al.,* 1990b; Flynn *et al.,* 1994; Bechemin *et al.,* 1999; John and

Flynn, 2000). In view of the involvement of P-rich DNA in cell division, Anderson *et al.* (1990b) proposed that P limitation could cause an increase in the availability of intracellular arginine, due to reduced demand from competing P-dependent pathways involved in cell division. Arginine has been shown to be a precursor in PST biosynthesis (Shimizu, 1996). It follows that intracellular arginine concentrations would be positively correlated with cellular toxin content. Indeed, Flynn *et al.* (1996) reported that toxin synthesis in *A. minutum* was proportional to intracellular concentrations of amino acids, arginine in particular. Conceivably, P limitation may cause cell division to cease but allow the cells to continue synthesizing PSTs or convert other cellular constituents to the toxins.

D. Organic Nutrients

Studies on the effects of organic nutrients on toxin production in dinoflagellates (Levasseur *et al.,* 1995; John and Flynn, 1998, 1999; Hwang and Lu, 2000) have revealed that the effects are rather insignificant because almost all PST-producing dinoflagellates are photoautotrophs. Although two *Alexandrium* species, *A. tamarense* and *A. fundyense*, seemed able to take in dissolved free amino acids at environmentally realistic levels (n*M*) during exponential growth, there was generally no apparent enhancing effects on toxin synthesis by unnaturally high levels of amino acids. Urea was found un-utilizable by *A. tamarense* (cf. *excavatum*), indicating the lack of urea catabolism activity in this species (Levasseur *et al.,* 1995). Hwang and Lu (2000) found that humic acids actually inhibited the growth of *A. minutum*. In our own lab, we found that humic acids and mixtures of phytohormones (6-benzylaminopurine, indole-3-acetic acid, kinetin) had little or detrimental effects on PST bioproduction.

E. Trace Metals

Little is known of the role of trace metals for toxin biosynthesis. In one study in which *Alexandrium tamarense* was cultured under iron-limiting conditions, cellular toxin content was only slightly higher than that of the control cultures in the early stationary phase (Boyer *et al.,* 1985). Any effect is probably indirect, through interference with the photosynthetic apparatus, including the Fe proteins, ferredoxin, and cytochromes. In our lab, we have found similar results with iron as well as with manganese and cobalt on the toxin production of *Alexandrium tamarense*. By adjusting the K-medium formula (Keller *et al.,* 1987), the toxin production of cultures increased slightly by decreasing iron

and manganese concentrations and increasing cobalt concentrations. However, there was always a concomitant increase in the cell number, meaning that the toxin per cell was virtually unchanged under all concentrations used. Other investigators have looked at the requirement of selenium for growth by *A. minutum* and *G. catenatum,* but did not examine the effects on toxin production (Doblin *et al.,* 1999). Evidently, further studies need to be done to determine the optimum concentrations necessary for maximum toxin yield.

IV. Environmental Factors

Effects of environmental factors on PST production in dinoflagellate cultures have been extensively investigated (Boyer *et al.,* 1987; Ogata *et al.,* 1987; Anderson *et al.,* 1990b; Flynn *et al.,* 1994, 1996; Ogata *et al.,* 1996; Mastuda *et al.,* 1996; MacIntyre *et al.,* 1997; Hwang and Lu, 2000). Especially important factors include salinity, temperature, light, and mixing disturbance. These factors affect the physiology and reproduction of the dinoflagellates and therefore their secondary biosynthetic activities as well.

A. Salinity

Salinity is known to affect nutrient uptake and a variety of active and facilitated transport systems of dinoflagellate cells. There is no fixed optimal salinity of the medium for PST production in algal cultures. White (1978) found that sufficient medium salinity was required for high cellular toxin content of *Alexandrium excavatum*. Similarly, in a toxic clone of *A. tamarense,* the highest cell toxicity occurred when the culture was at the highest salinity-dependent growth rate in the exponential phase (Parkhill and Cembella, 1999). Recently, Hwang and Lu (2000) also observed that good production of GTX 2 and 3 in *Alexandrium minutum* required a sufficiently high level of salinity. However, Usup *et al.* (1994) found that high salinity was detrimental to toxin production in *Pyrodinium bahamense*, while others (Anderson *et al.,* 1990b; Flynn *et al.,* 1996) did not find any significant effects of salinity on toxin production in some *Alexandrium* species within a range. It is concluded that there is a considerable range of salinity within which PST production is not significantly affected as long as algal growth remains unchanged.

B. Temperature

There have been consistent observations that high temperatures for elevated growth are correlated with reduced PST productivity in

dinoflagellates (Ogata *et al.,* 1987; Usup *et al.,* 1994; Hwang and Lu, 2000). These observations support the notion that cell division (growth) and toxin biosynthesis are two competing and mutually antagonizing processes. The direct effect of temperature on PST biosynthesis is not clear. In *Alexandrium* species, low cellular toxin content found at an elevated temperature was invariably associated with an increased growth rate (Proctor *et al.,* 1975; Ogata *et al.,* 1987). Similarly, Usup *et al.* (1994) concluded that the reduced toxin production in *Pyrodinium bahamense* at an elevated temperature was due to an increased mean cell division rate that was temperature dependent. Anderson *et al.* (1990b) more specifically postulated that increased cellular toxin content at low temperatures reflects an allocation of cellular nitrogen, especially intracellular arginine, in favor of PST biosynthesis over the biosynthesis of cellular protein. Thus, we conclude that temperature should be adjusted such that growth and toxin production are balanced to produce the maximum amount of toxin.

C. Light

Light is of primary importance in the growth and toxin production of toxic dinoflagellates because most of them are photoautotrophic. Sufficient photo energy is required for the cells to fix carbon dioxide and harness the light energy for metabolism and biosynthesis. Production of oxygen for respiration as a result of photosynthesis also plays a significant role in algal growth and reproduction. In *Pyrodinium bahamense*, cellular toxin content decreased with reduced light intensity (Usup *et al.,* 1994). Ogata *et al.* (1987) concluded that photosynthesis was essential for toxin production in *Alexandrium tamarense*. This is understandable considering that the energy cost of toxin biosynthesis would be undertaken at the expense of photosynthetically derived carbon compounds (e.g., acetate and amino acids) as well as energy-rich intermediates (e.g., ATP). Based on the observations of these and other investigators (Parkhill and Cembella, 1999; Hwang and Lu, 2000), it can be assumed that sufficient light energy is required for maximum toxin yield.

D. Aeration and Agitation

Dinoflagellate cultures are generally considered sensitive to agitation which may cause growth reduction and cellular damage (White, 1976; Galleron, 1976; Tuttle and Loeblich, 1975)—hence, aeration is seldom used in culturing dinoflagellates due to the unavoidable agitation caused. However, our recent studies indicated that gentle circulation

produced by optimized aeration enhanced both the growth and toxin production in the culture of *Alexandrium tamarense* used (Wang and Hsieh, 2001). It was noted that the size of air bubbles was an important operational parameter. Large bubbles ($D \geq 3$ mm) were detrimental to culture productivity, probably due to the higher turbulence and shearing forces produced and a relatively inefficient gas-to-liquid transfer of CO_2. Apparently, aeration increased the supply of CO_2 needed for growth and stabilized the medium pH through the buffering capacity of the carbonate/bicarbonate system. A pH-stat system would have provided a finer control of the pH as well as the concentration of inorganic carbon for photosynthetic uptake. Enhancement of both the biomass and toxin yield through stabilization of medium pH was also observed in a number of other studies (Goldman *et al.,* 1982; Guillard and Keller, 1984; Babinchak *et al.,* 1994).

V. Prototype Laboratory Production of C2 Toxin

In the last two years, we have endeavored to investigate culture conditions optimal for PST bioproduction and thereby to produce milligram quantities of pure PST as reference compounds for toxicological studies and analytical methods development. Our efforts have resulted in the establishment of two culture techniques capable of producing two of the PST in a highly purified form, C2-toxin (C2T) and gonyautoxin 3 (GTX3).

A. Organism

A local strain of *Alexandrium tamarense* (ATCI01), isolated from a single cyst from the Dapeng Bay, South China Sea (Jiang *et al.,* 2000), was used for our bioproduction studies and operations. The original strain was maintained in the Institute of Hydrobiology, Jinan University, Guangzhou, PRC. A subculture of the strain, obtained from Prof. Y. Qi of the Institute, has been maintained in our laboratory since 1998 at 23°C with 5000 lux and a 14/10 h light/dark cycle in natural seawater K medium (Keller *et al.,* 1987) in standing cultures. The seawater used was collected at the Port Shelter near the Hong Kong University of Science and Technology, and was filtered through 0.2-μm Millipore filters into polypropylene carboys and stored in the dark until used. Of the suite of PST known, we found that this strain produces predominantly C2T at over 99% on a mole basis (Hsieh *et al.,* 2001). This toxin profile is distinctly different from those reported for the same species from other geographical areas (Ogata *et al.,* 1987; Kim *et al.,* 1993; Levasseur *et al.,*

1995). C2T and its metabolites may very well be a distinct feature of PST contamination in Hong Kong coastal areas, which warrants further investigation. The use of natural seawater was necessary because this strain began to lose its toxigenicity in an artificial seawater medium after a few cycles of subculturing.

B. Culture Techniques and Conditions

In our endeavors, we examined the toxin production of ATCI01 in batch cultures of various sizes, ranging from 50 ml to 40 liters. After manipulating nutritional and environmental conditions, mentioned in Sections III and IV, we established an optimal set of conditions for large-scale toxin production operations. The conditions of an actual preparative batch culture are summarized in Table III.

Having established large-scale batch cultures and found that the toxin yield peaked in the stationary phase (see the following section), we designed a combined semicontinuous culture and temporary (holding) culture system for mass production of C2T (Wang *et al.,* 2001). This system divides the culturing process into growth and production stages to maximize both biomass and toxin yield.

Algal cells are first cultivated in semicontinuous cultures to achieve high cell concentrations; this constitutes the growth stage. In this manner, a continuous supply of biomass for toxin biosynthesis is produced. For the toxin production stage, portions of the growing culture are removed and transferred to holding chambers under phosphate deplete conditions optimal for C2T biosynthesis in the cells. The culture conditions of this system that were actually used are summarized in Table IV. By this design, cells with high toxin content were continuously harvested for toxin extraction and purification.

TABLE III

The Conditions of a Batch Culture of *A. Tamarense* ATCI01 For Mass Production of C2T

Conditions/parameters	Data/information
Culture volume	20 liters in 70 liter rectangular tanks
Medium	Natural seawater K medium ($PO_4 = 10\ \mu M$)
Inoculum size	5% at 10^4 cells/ml
Incubation conditions	23°C, 8000 lux, 16/8 (L/D), aerated (<2 mm bubbles)
Peak cell concentration	15,000–20,000 cells/ml in 6 days
Harvesting time	10 days
Toxin yield	480–560 μg/liter of C2T

TABLE IV

THE CONDITIONS OF A COMBINED SCC AND TEMPORARY CULTURE SYSTEM OF *A. TAMARENSE* ATCI01 FOR MASS PRODUCTION OF C2T

Conditions	Semicontinuous	Temporary
Culture volume (liter)	40	40
Medium	Natural seawater K medium ($PO_4 = 5\mu M$)	Spent medium lacking PO_4
Duration	Day 0–5 as batch culture Day 6–18 as SCC	6 days
Dilution rate	0.5 per 3, 2, and 1 day	
Incubation conditions	23°C, 8000 lux, 16/8 (L/D), aerated	23°C. 8000 lux, 16/8 (L/D), aerated and nonaerated
Peak cell concentration (cells/ml)	18,000 (0.5/3 day) 17,000 (0.5/2 day) 16,000 (0.5/1 day)	15,000
C2T yield (μg/liter)	320 (0.5/3 day) 280 (0.5/2 day) 320 (0.5/1 day)	496

C. GROWTH AND TOXIN PRODUCTION

The dynamics of algal growth and PST production in the two culture designs of *A. tamarense* we used are summarized as follows.

1. *Batch Culture*

In a 20-liter nutrient-replete batch culture of ATCI01, the cells grew exponentially between day 1 and 6, with a growth rate of 0.56 divisions/day and a peak cell concentration of approximately 15,000 cells/ml. A distinct stationary phase was seen after day 6 due to phosphate depletion. During the stationary phase, the cell density remained high and relatively constant. The average diameter of actively growing cells was about 25 μm but reached up to 50 μm when the cells were well into the stationary phase. The growth profile is shown in Figure 2.

The toxin yield, measured in micrograms of C2T per liter of culture (μg/liter), increased rapidly during the early exponential phase and continued to increase during the stationary phase. It reached a peak of 512 μg/liter on day 10 and then declined to about 320 μg/liter on day 15 (Fig. 2). The specific cellular C2T content (pg/cell) was at a low level during the exponential phase when the cells were small. It increased rapidly during the early stationary phase and reached a peak of about 36 pg/cell on day 10 when the cells doubled in diameter. It then decreased to about 24 pg/cell on day 15.

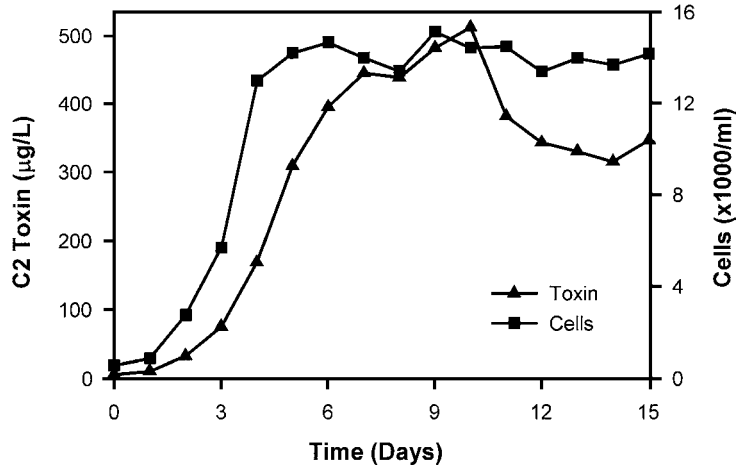


FIG. 2. The growth and C2T production profiles of *A. tamarense* ATCI01 in a 20-liter batch culture.

The phosphate concentration in the culture medium decreased rapidly and was depleted on day 4, constituting a phosphate-limiting condition for the culture from day 4 onward. The pH of the culture medium, initially at 8.2, rose with cell growth to a peak of 9.1 and then declined to 8.4 by day 15.

The strain ATCI01 and the aforementioned culturing methods were successfully used for mass production of C2T for purification and further studies. A portion of the C2T produced was also hydrolyzed under acidic conditions to obtain GTX3 (Fig. 3).

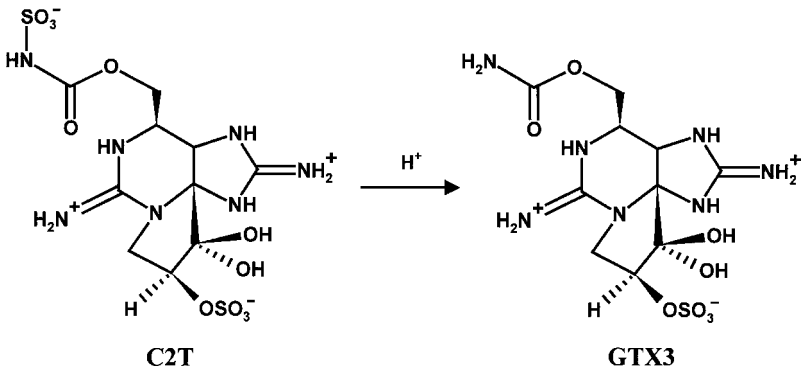


FIG. 3. Chemical conversion of C2T to GTX3.

It should be noted that in the batch cultures of ATCI01, significant amounts of C2T were found in the culture medium. Analysis of the seawater showed that over 200 μg/liter of C2T was found in the culture medium, which is similar to observations we have made previously (Hsieh *et al.,* 2001). This indicated that the toxin had come out of the cells. The "extracellular" toxins might have been excreted by the cells, leaked through the aged and weakened cell surfaces, or released from lysed cells. Whether PST are exotoxins needs further verification. Regardless of the explanation for the presence of toxins in the medium, for our production operations, we did not attempt to recover the toxins in the medium due to the lack of an efficient method to collect and recover the toxins in the spent aqueous medium.

2. *Combined Semicontinuous Culture and Temporary Culture*

For the combined culture system, a 40-liter batch culture was started under the same conditions as described for batch cultures above for the first five days until the cell density peaked. Thereafter, a semicontinuous culture (SCC) scheme was initiated and maintained. One 3-day dilution cycle, two 2-day dilution cycles, and three 1-day dilution cycles were used for the SCC. For each cycle, 20 liters of the culture was transferred upon mixing to the temporary culture tank and replaced with an equal volume of fresh growth medium. Both cultures were again aerated with small air bubbles of less than 2 mm in diameter. The cell concentration in SCC reached 15,000 cells/ml when the 3-day dilution cycle began at day 5. Throughout the various dilution cycles, the peak cell-concentrations at the end of each dilution cycle averaged around 17,000 cells/ml. The corresponding peak C2T contents in the SSC ranged from 200 to 320 μg/liter for the various cycles (Table IV). In the temporary culture, the cell concentration was maintained at around 15,000 cells/ml in an established stationary phase. From the onset of the temporary culture, the toxin content steadily increased as expected during the first 4 days to peak at 500 μg/liters and then declined to 240 μg/liter by day 6. We harvested the algal cells in the temporary culture at day 4 for extraction of C2T. Thus we used this two-stage system to capitalize on the high cell biomass in the SCC and the high cellular toxin content in the temporary culture to maximize the C2T yield. It must be noted that aeration was important to maintain both a higher cell number and toxin yield.

D. Cell Harvesting and Toxin Extraction

Algal cells were harvested by two methods for toxin extraction: filtration and centrifugation. For small volumes both are satisfactory,

but for mass production, filtration has the problem of clogging of filters while centrifugation alone is only possible if a continuous centrifuge is available. In our production operation, cells to be harvested (in the late stationary phase in day 12–14) were allowed to settle on the bottom of the culture tanks, and then most of the supernatant medium containing very few cells was removed. The remaining highly concentrated culture was collected and centrifuged at 3000 rpm for 20 min. The resulting cell pellet was resuspended in 50 m*M* acetic acid and stored at −70°C until further processed.

The frozen cells were ruptured by freeze-thawing three times followed by sonication on ice for 1 min and agitation in an ultrasonic bath for 15 min to release the toxins from the cells. The cell homogenate was centrifuged to obtain the supernatant containing the toxin. The toxin extract was concentrated under vacuum and then stored at 4°C until purified.

VI. Toxin Purification and Analyses

The concentrated crude toxin extracts were passed through a 10,000 molecular weight cut-off filter to remove proteins and other large molecules. The filtrate was concentrated and fractionated through a series of chromatography columns, including P2 gel, C18, and again P2 gel. In this manner, milligram quantities of C2T was purified, after which, a portion was converted to GTX3.

The purified toxins were analyzed by high pressure liquid chromatography and post-column reaction system (HPLC-PCRS) with fluorescence detection following the method of Oshima (1995) using a column temperature of 30°C and a reaction coil temperature of 45°C as modified by Anderson *et al.* (1996). Their purity and structures were confirmed by HPLC, mass, and nuclear magnetic resonance (NMR) spectrometric analyses (Lin and Hsieh, 2001). C2T, whose analytical standard is currently unavailable, was quantified by stoichiometric conversion to GTX3 followed by HPLC analysis against a GTX3 standard purchased from NRC, Canada. In addition, the C2T quantity was further verified by NMR analysis using *tert*-butanol as an internal standard.

VII. Conclusion

Based on our results and those of others, it is feasible to produce milligram quantities of PSTs as a supply of the pure toxins for R&D and monitoring studies using the algal culture approach. Among factors that significantly influence toxin productivity are species of dinoflagellates, culture designs, as well as nutritional and environmental conditions. Optimization of these factors to maximize the toxin yield is largely

empirical at this stage because the mechanisms of action of these factors on the algal growth and toxin biosynthesis are not sufficiently known. Nonetheless, it is evident that C, N, and P are crucial nutritional factors, while light, temperature, and aeration are important physical conditions that must be regulated in order to obtain high yields of PSTs. The combined semicontinuous culture and temporary culture described in this article offers a cost-effective system for laboratory bioproduction of C2T. This system is amenable to scale up for mass production and modification for the production of other PSTs.

Acknowledgments

We want to gratefully acknowledge the financial support received from the following extramural agencies for our research on marine biotoxins: the Pearl River Estuary Pollution Project funded by the Hong Kong Jockey Club, the Hong Kong Environmental Conservation Fund and the Croucher Foundation.

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Metal Toxicity in Yeasts and the Role of Oxidative Stress

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I. Introduction

Metal pollutants are generated through a wide range of industrial activities and continue to be released into the environment at potentially harmful levels. Localized concentration of certain metals may also arise naturally. For example, toxic levels of the biologically essential metal copper are often associated with certain mineral ores as well as industrial or agricultural discharges. The biologically inessential metal cadmium is used widely in electroplating and galvanizing industries, as a color pigment in paints and in batteries, and as a by-product of zinc and lead mining and smelting. Zinc, lead, and other metals also may be released from similar types of sources, and the pollution that can ensue continues to provoke serious concern among environmental groups and the medical community. Such concern is exacerbated by recent reports that pollution with metals (e.g., cadmium) and associated toxicity in natural populations is considerably higher than previously thought (Larison *et al.,* 2000).

The interactions of yeasts and other microorganisms with metals have been of scientific interest for some time. Free metals and compounds

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0065-2164/01 $35.00

that contain complexed metals, such as copper, have been used widely in fungicides, bactericides, and algicides. Metal toxicity continues to be exploited for many fungicides used in the control of plant pathogens and for material preservation (Gadd, 1993). Furthermore, metal complexation is being used to enhance the effectiveness of certain antifungal drugs (Nicoletti *et al.,* 1999; Mastrolorenzo *et al.,* 2000). Organisms subject to routine metal exposure in their natural environments generally have had to develop resistance mechanisms. Weissman *et al.* (2000) recently proposed that the elevated copper resistance of the yeast pathogen *Candida albicans* might reflect an adaptation to Cu in the gastrointestinal tract. Silver resistance may also enhance the prevalence of *C. albicans* as a nosocomial pathogen (Riggle and Kumamoto, 2000). In an applied context, the ability of certain yeasts and fungi to resist metal toxicity, combined with their generally high metal-absorptive capacities, has been exploited in the development of certain biological metal-removal processes (White *et al.,* 1998; Gadd, 2000). Furthermore, to address the need to monitor environmental levels of toxic metals, yeasts such as *Saccharomyces cerevisiae* are being used in efforts to develop novel metal-ion biosensors (Lehmann *et al.,* 2000).

As well as the relevance of metal toxicity to environmental/industrial applications of yeasts and other fungi, these and other microorganisms also serve as useful models for characterizing certain aspects of metal toxicity in higher organisms. In humans, metals are implicated in a wide range of degenerative conditions, including neurotoxicity, nephrotoxicity, genotoxicity, and carcinogenesis (Halliwell and Gutteridge, 1990; Stohs and Bagchi, 1995; Campbell and Bondy, 2000). The requirement to maintain an appropriate homeostatic balance of essential metals such as copper is reflected by the prevalence of Menkes syndrome, associated with Cu deficiency, and Wilson's disease, associated with excess Cu accumulation (Harris and Gitlin, 1996). Obviously, metal toxicology in higher eukaryotes is an issue of significant concern and there has been considerable effort to characterize the effects of metals that are manifest at the tissue, organ, or whole-organism level. Unfortunately, these foci have tended to divert attention from mechanisms of metal toxicity acting at the cellular or molecular level, which ultimately lead to tissue and organ damage. There is a recognized need to redress this balance, i.e., to provide a detailed understanding of the molecular/cellular targets of metal action and of the underlying mechanisms involved (Waalkes, 1995; Nieboer and Fletcher, 1996). One reason for the current lack of understanding of these aspects is the difficulty of studying them in higher organisms, which has lead to a partial reliance on information from potentially nonextrapolatable *in vitro* work. *Saccharomyces cerevisiae* and other yeasts are receiving increased attention as experimental cell

systems that may provide a better reflection of the cellular effects of metals *in vivo* (Sect. IV).

This review provides an overview of our current understanding of metal toxicity in yeasts, with particular emphasis on the mechanisms and targets of metal action. Since many potential targets of metal toxicity are common to more than one metal, the review is organized such that different targets rather than different metals are dealt with separately. Nonetheless, the differing biochemical properties of metals cannot be disregarded, particularly since these properties generally dictate the molecular interactions underpinning macromolecular damage. Hence, such interactions for specific metals are addressed wherever pertinent, particularly in Section II. For more detailed accounts of the chemistries of specific toxic metals, the reader is referred to general chemistry texts, as well as other reviews (e.g., Hughes and Poole, 1991; Gadd, 1992). The principal intention here is to review the evidence from studies with yeasts into how metals exert their toxic effects in cells. One of the main hypotheses that will be discussed, and in most part supported, is that metal toxicity is a consequence of metal-induced free radical generation.

II. Metal-Induced Generation of Reactive Oxygen Species (ROS)

It has been known for some time that many metals can promote the generation of highly damaging free radicals, or reactive oxygen species (ROS), in biological systems. There are several possible mechanisms of metal-induced ROS formation (see below), proposed largely through evidence from *in vitro* studies. However, the physiological consequences of metal-induced ROS generation *in vivo* are less well characterized. This is a serious issue since the number of harmful effects that ROS are considered to provoke in micro- and macroorganisms is large, and continues to increase (for reviews, see Fridovich, 1998; Halliwell and Gutteridge, 1999; Finkel and Holbrook, 2000). Major cellular macromolecules such as lipids, proteins, and DNA are highly susceptible to oxidative attack and degradation (ROS attack on carbohydrates is comparatively slow). Reasons for elevated cellular ROS levels are severalfold: as well as intrinsic alterations in host physiology, extrinsic stress (e.g., metal toxicity) is a commonly cited cause.

Metal-induced ROS formation could occur via several mechanisms. The Fenton or Haber–Weiss reactions (see Fig. 1) are catalyzed by redox-active metals (e.g., Cu, Fe, Cr, V) and generate the highly reactive hydroxyl ($OH^{\bullet}$) radical from H_2O_2 and superoxide ($O_2^{\bullet -}$) substrates (the latter being formed during respiration) (Halliwell and Gutteridge, 1999). Cu and Fe may also initiate oxidative damage directly, e.g., by promoting

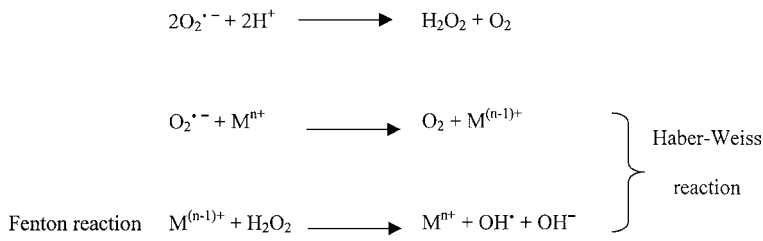

FIG. 1. ROS generation in cells. The Fenton and Haber–Weiss reactions are catalyzed by redox-active metals.

the propagation reactions of lipid peroxidation. Several indirect mechanisms are considered to account for the action of redox-inactive metals such as cadmium, mercury, nickel, and lead. For example, these metals might displace redox-active metals from cellular binding sites (Casalino *et al.,* 1997). Indeed, the toxicity of $O_2^{\bullet -}$ is believed to be mediated indirectly in a similar manner, via release of Fenton catalysts from certain iron–sulfur [4Fe-4S] enzymes (Srinivasan *et al.,* 2000). Further potential indirect mechanisms by which metals may promote ROS damage include inhibition of antioxidant defense enzymes (Sarkar *et al.,* 1997; Murakami and Yoshino, 1999), depletion of protein-bound sulfhydryl groups and glutathione (Stohs and Bagchi, 1995; Fortuniak *et al.,* 1996; FigueiredoPereira *et al.,* 1998), and physical perturbation of membrane lipid arrangement with concomitant sensitization to lipid peroxidation (Verstraeten and Oteiza, 1995). The result of each of these processes would be a tendency toward elevated ROS formation and/or increased lipid, protein, and DNA oxidation. Indeed, in support of a role for free radical reactions in metal toxicity, there are close similarities between the gross effects of oxidative damage and metal damage on cellular macromolecules.

A. Lipid Peroxidation

The net result of lipid peroxidation is conversion of unsaturated lipids to polar lipid hydroperoxides that can cause increased membrane fluidity due to disturbance of hydrophobic phospholipids (Dix and Aikens, 1993; Van Ginkel and Sevanian, 1994). Metal stress is also associated with increased membrane fluidity (Assmann *et al.,* 1996; Howlett and Avery, 1997a). Such effects can profoundly influence membrane functions. For example, lipid peroxidation, like metal stress, may be accompanied by membrane permeabilization with efflux of cytosolic solutes (Van Ginkel and Sevanian, 1994; Howlett and Avery, 1997a,b). Extensive lipid peroxidation can lead to the ultimate disintegration of membrane integrity and possible cell death. It has yet to be confirmed

unequivocally that metal-induced lipid peroxidation is the cause of such macroscopic effects during metal toxicity.

B. Protein Oxidation

Proteins display differing susceptibilities to oxidative attack, which may be linked to variable compositions of sulfhydryl groups, Fe–S clusters, reduced heme moieties, and Cu prosthetic groups (Davies, 1995; Strain *et al.,* 1998). Certain amino acids (e.g., Cys, Met, His) are also particularly prone to oxidation. Accumulation of oxidized amino acid residues leads to protein conformational changes and inactivation (Stadtman and Berlett, 1997), similar to the reported effects of metals (Hughes and Poole, 1991; Jungmann *et al.,* 1993a). A probable role of oxidative damage in metal effects on proteins is reflected in the sensitivity of certain proteins to both oxidative and metal stress (Dubey and Rai, 1987; Watabe *et al.,* 1995). Such effects may result from site-specific ROS damage at sites of metal (e.g., Fe, Cu) binding (Halliwell and Gutteridge, 1999). Nonspecific metal-induced ROS generation may also affect proteins, for example by causing side-chain modifications (Davies, 1995). In addition, membrane proteins may be oxidized by products of (metal-induced) lipid peroxidation.

C. DNA Oxidation

Oxidative damage to DNA includes base modifications, DNA–protein cross links, depurination, and strand scission (Beckman and Ames, 1997; Wallace, 1997). These effects are very similar to those observed during metal exposure (Waalkes, 1995; Kasprzak, 1996; Lloyd *et al.,* 1997). Mutagenicity arises from erratic replication and/or repair of the damaged DNA. When cellular DNA repair mechanisms are overwhelmed, extensive DNA damage can lead to cell death.

III. General Evidence for Involvement of ROS in Cellular Metal Toxicity

At the macroorganism level, there has been growing evidence for a key involvement of oxidative stress in metal-related damage and disease (for reviews, see Halliwell and Gutteridge, 1990; Rotilio *et al.,* 1995; Kasprzak, 1996; Hippeli and Elstner, 1999; Campbell and Bondy, 2000). Much of this evidence is less circumstantial than that based on the similarities between ROS and metal effects (above). Thus, elevated oxidation of cellular components (e.g., lipid peroxidation) is commonly reported in metal-exposed cells and animals (e.g., De Vos *et al.,* 1993; Sarkar *et al.,* 1997; Bagchi *et al.,* 1998; Baryla *et al.,* 2000) and protection may be conferred by antioxidants (e.g., Sarkar *et al.,* 1997;

Pourahmad and O'Brien, 2000). The timings of events associated with oxidative damage (e.g., lipid peroxidation) may also be correlated with the gross macromolecular damage caused by metals (e.g., membrane permeabilization), although such evidence is inconclusive since detection of gross damage may require prolonged accumulation of oxidative damage (Howlett and Avery, 1997a). The cumulative evidence shows that many toxic metals promote ROS formation in cells. However, in most cases it has not been clearly resolved whether ROS-mediated effects are responsible for metal-induced cell injury, or if they are merely incidental.

This issue is complicated further by a number of factors. First, certain potentially toxic metals also have antioxidant properties. For example, zinc deficiency is considered to enhance the susceptibility of plants to attack by ROS (Cakmak, 2000) and Zn suppresses Cd-induced apoptosis in higher cells, which is believed to be in part due to the antioxidant action of Zn (Szuster-Ciesielska *et al.,* 2000). Nramps (natural resistance-associated macrophage proteins), which are found in many organisms, are believed to protect cells against ROS damage by sequestration of Mn(II) and Fe(II) as cofactors for antioxidant enzymes (Nelson, 1999; Kehres *et al.,* 2000). Copper can also confer protection against ROS (Section VI.A.5). Second, redox-active metals such as Fe(II) and Cu(I) may be toxic under both aerobic and anaerobic conditions (Strain and Culotta, 1996; Dunning *et al.,* 1998). This issue with regard to yeasts is discussed below (Sect. VI.A.2). Third, the relative importance of free radical damage in metal toxicity is likely to be metal dependent. Certain metals are not known to promote ROS formation at all, and exert their toxicities through alternative mechanisms, e.g., Cs^+ displaces cellular K^+, apparently causing K^+ starvation (Avery, 1995). Fourth, ROS-independent toxicity mechanisms are known even for metals that are prime candidates for ROS-dependent toxicity. For example, in addition to effects dependent on the redox activity of copper, Cu toxicity could be mediated through inappropriate binding of the metal to N, O, and S ligands in biomolecules, thereby inactivating enzymes and disrupting organism function (Karlstrom and Levine, 1991). Furthermore, it has been demonstrated that several metals can replace Zn in the zinc finger DNA binding domain of the human estrogen receptor, resulting in a protein that no longer can bind to its target sequence (Predki and Sarkar, 1992).

As mentioned earlier, it is recognized that one main reason for the above ambiguity and the general poor understanding of metal toxicology at the cellular or molecular level is the difficulty of studying such processes in higher organisms with a consequent overreliance on information from potentially nonextrapolatable *in vitro* work (Stohs and Bagchi, 1995; Kasprzak, 1996; Klein, 1996). The yeast *Saccharomyces*

cerevisiae provides an excellent alternative experimental system for elucidating the underlying cause(s) of cellular metal toxicity, a matter that is now essential for advancing our understanding in the field of metal toxicology.

IV. The Yeast Model Applied to Metal Toxicology

For the reasons given in the Introduction, an understanding of metal toxicity is directly pertinent to many aspects of yeast and fungal biotechnology, pathogenesis, and general physiology. Therefore, it is fortuitous that yeasts, particularly *S. cerevisiae,* provide outstanding experimental systems with which such understanding can be attained. The ease with which *S. cerevisiae* can be manipulated facilitates detailed elucidation of many aspects of eukaryotic cell physiology and/or toxicology that would not be convenient with filamentous fungi and higher eukaryotic systems. The genetics of *S. cerevisiae* are very well understood and the completion of its genome sequence in 1996 has facilitated recent strides in functional genomics technologies applied to *S. cerevisiae* (e.g., Spellman *et al.,* 1998; Winzeler *et al.,* 1999; Uetz *et al.,* 2000). Furthermore, there has been striking conservation of cellular function through evolution between *S. cerevisiae* and mammalian cells, and the similarities are exploited heavily to provide insight into cellular processes that occur in humans and other organisms. With regard to metal toxicity, further advantages of the yeast model are as follows: (a) most of the reported effects of metal toxicity (Halliwell and Gutteridge, 1990; Gadd, 1993; Perego and Howell, 1997; Naganuma *et al.,* 2000) and responses to oxidative stress (Santoro and Thiele, 1997; Grant *et al.,* 1998; Jamieson, 1998; Sigler *et al.,* 1999) are common to yeast and animal cells; (b) extensive information on general aspects of yeast–metal interactions and the genetic regulation of metal homeostasis and oxidative stress adaptation are available for *S. cerevisiae* (see Gadd, 1993; Santoro and Thiele, 1997; Askwith and Kaplan, 1998; Jamieson, 1998; Labbe and Thiele, 1999; O'Halloran and Culotta, 2000); (c) several valuable methods not applicable to higher organisms can be used with yeasts for characterizing cellular stress or damage (Halliwell and Gutteridge, 1999); and (d) the genetic basis of oxidative DNA damage and repair are very well described in *S. cerevisiae* (Girard and Boiteux, 1997). Hence, as well as the pertinence of yeast/fungal–metal interactions in an applied context, *S. cerevisiae* provides a well-described eukaryotic framework on which to build a detailed understanding of cellular metal toxicology.

The focus of the remainder of this review is almost exclusively on yeasts, although there will be occasional reference to studies with other

organisms where appropriate. *S. cerevisiae* is cited most, simply because the vast majority of yeast–metal studies have been performed with this organism. However, pertinent studies with other yeasts (e.g., *Candida, Schizosaccharomyces, Hansenula* spp.) are discussed wherever possible.

V. General Aspects of Metal Toxicity and Resistance in Yeasts

The interactions of yeasts with toxic metal ions have been studied extensively in recent years. Many potentially toxic metals are essential for normal metabolism at physiological concentrations, e.g., Cu, Zn, Fe, Mn. Therefore, cells have developed elaborate mechanisms for regulating the cellular content and compartmentalization of these essential metals. Such mechanisms in *S. cerevisiae* are the subject of current study, but will not be dealt with here since they are only indirectly relevant to providing an understanding of metal toxicity. Metal homeostasis in *S. cerevisiae* is covered in excellent reviews elsewhere (e.g., Askwith and Kaplan, 1998; Labbe and Thiele, 1999; O'Halloran and Culotta, 2000). Nonetheless, studies of metal homeostasis have yielded some important findings that are pertinent here. For example, owing to the efficiency of cellular Cu chaperones, it has been estimated recently that the total cytoplasmic free copper content of *S. cerevisiae* is many orders of magnitude less than one atom per cell (Rae *et al.,* 1999). This underscores the difficulty of interpreting total cellular metal contents in toxicological terms, as also applies to extracellular metals where bioavailability may be diminished by ligand binding and precipitation (Hughes and Poole, 1991; Avery and Tobin, 1993; Gadd, 1993). This finding also underscores the potency of metals such as copper. Considering such potency among potentially toxic metals, it is surprising that relatively few studies have sought specifically to elucidate the mechanism(s) of cellular metal toxicity with yeasts, whereas many studies have focused on the mechanisms used by yeast cells to evade such toxicity. This is not to say that a detailed knowledge of resistance mechanisms cannot, in turn, contribute to our understanding of toxicity. The remainder of this section gives an overview of yeast metal tolerance/resistance mechanisms. How these may pertain to our understanding of metal toxicity is addressed in subsequent sections.

The ability of yeasts to survive in the presence of toxic-metal species hinges on an array of specific and nonspecific tolerance/resistance mechanisms. As in other microorganisms (Nies, 1999), certain yeast activities may promote external metal complexation through reactions that are believed to contribute to metal tolerance. For example, the *SLF1* gene product is important for the process of copper sulfide (CuS) precipitation at the cell surface of *S. cerevisiae* grown in copper-containing

medium and diminishes Cu toxicity (Yu *et al.,* 1996). Other such reactions including extracellular complexation, metal transformation (e.g., oxidation, reduction, methylation, dealkylation), biosorption to cell walls, pigment and extracellular polysaccharide release, together with decreased transport or impermeability, are often considered intrinsic (nonadaptive) processes that may confer varying degrees of metal tolerance (see Gadd, 1993; Cervantes and Gutierrez-Corona, 1994). Mechanisms that act intracellularly often are inducible by metal stress and it is perhaps more appropriate to term these "resistance" mechanisms (Gadd, 1993).

Most cellular metal-resistance mechanisms alter intracellular metal availability—for example via efflux, intracellular compartmentation, complexation, and/or precipitation. Several metal efflux systems have been characterized in yeasts. Arsenical resistance in *S. cerevisiae* is mediated by arsenite extrusion via Acr3p, following arsenate reduction by Acr2p (Mukhopadhyay *et al.,* 2000). Efflux activity also enhances the Cd resistance of *S. cerevisiae* strains carrying the *CAD2* gene. *CAD2* is a mutated form derived from the gene of a putative Cu-transporting ATPase (*PCA1*), and is proposed to act as a Cd efflux system (Shiraishi *et al.,* 2000). The Cu-transporting P-type ATPase, Cacrp1p (Crd1p), of *C. albicans* is noteworthy since it accounts for the greater Cu resistance of this organism compared to *S. cerevisiae* (Riggle and Kumamato, 2000; Weissman *et al.,* 2000). The same protein also confers some resistance to Ag and Cd (Riggle and Kumamato, 2000). Cacrp1p plays a more important role than Cu-metallothionein (Cu-MT) in the Cu resistance of *C. albicans* (Oh *et al.,* 1999; Riggle and Kumamato, 2000; Weissman *et al.,* 2000).

Metallothioneins are low molecular mass, cysteine-rich metal-binding proteins. In *S. cerevisiae*, Cu-MT encoded by *CUP1* is a key determinant of Cu resistance (Butt and Ecker, 1987; Mehra and Winge, 1991). When expressed at physiological levels in *S. cerevisiae*, Cup1p complexes and detoxifies Cu, and when overexpressed it can also protect against Cd. However, Cup1p does not protect against Ni, Pt, Co, or Zn. Copper-resistant strains of *Saccharomyces cerevisiae* contain 2–14 or more copies of the *CUP1* locus (Fogel *et al.,* 1983). A second metallothionein in *S. cerevisiae* is encoded by *CRS5*, but this plays a lesser role than Cup1p in copper detoxification (Jensen *et al.,* 1996). Like MT, the antioxidant molecule glutathione also is cysteine-containing and contributes to metal resistance in yeasts (Fig. 2). The actions of metallothionein and glutathione in metal resistance are discussed further below. The phytochelatins are γ-glutamyl peptides (effectively glutathione polymers) that confer metal resistance in *Schizosaccharomyces pombe* and other fungi, as well as algae and plants. *Schizosaccharomyces pombe* with a disruption in the *PCS* gene, which encodes

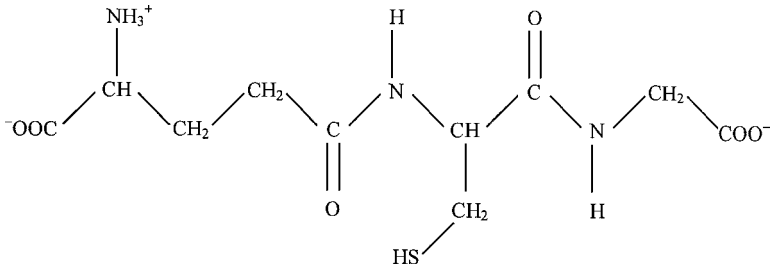

FIG. 2. Chemical structure of glutathione (GSH).

phytochelatin synthase, is hypersensitive to Cu and Cd (Clemens *et al.,* 1999). *Schizosaccharomyces pombe* synthesizes at least seven different phytochelatins in response to metals (Grill *et al.,* 1986).

In addition to metal efflux and complexation, intracellular metal detoxification may occur through vacuolar sequestration, which promotes resistance to a wide range of metal(loid) species (White and Gadd, 1986; Avery and Tobin, 1992; Ramsay and Gadd, 1997; Szczypka *et al.,* 1997; Gharieb and Gadd, 1998; Pearce and Sherman, 1999). In the vacuole, complexation with histidine may further diminish the availability of toxic metals (Pearce and Sherman, 1999). Complexation with amino acids affects both metal uptake and toxicity in *S. cerevisiae* (Joho *et al.,* 1990; Simmons and Singleton, 1996). The intracellular availability and toxicity of certain metals is also affected by competition from other less toxic ions (Perkins and Gadd, 1996; Blackwell *et al.,* 1998).

VI. Targets of Metal Toxicity in Yeasts and the Role of Free Radicals

A. Whole-Cell Metal Toxicity

The capacity of the above detoxification mechanisms to confer protection against toxic metals is of course limited. Toxicity will ultimately occur with elevations in metal concentrations. At the macroscopic level, almost any aspect of yeast or fungal physiology may be affected by metal toxicity. Harmful interactions proposed to occur at the molecular level, few of which have been demonstrated *in vivo,* include displacement of essential metal ions from (or blocking of) functional sites on biologically important molecules (e.g., enzymes, transport proteins), conformational modification of macromolecules, and membrane disruption (Gadd, 1993). Metal effects on the protein synthetic machinery may bring about cell cycle arrest (Philpott *et al.,* 1998). As mentioned above, in principle any of these interactions could either promote or be attributable to ROS formation (see Fig. 3). In instances where this is the

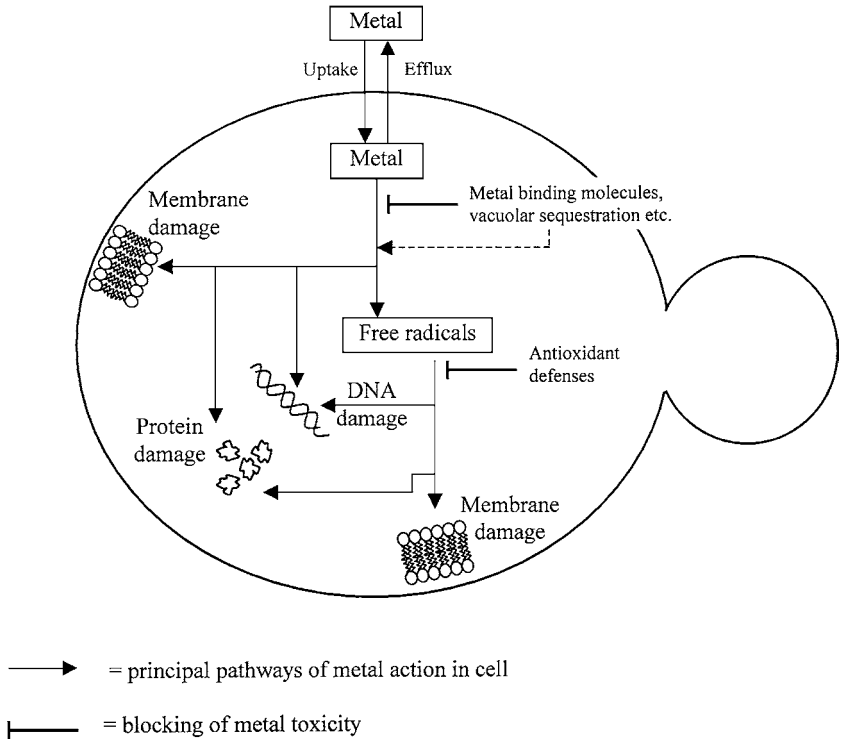

FIG. 3. Generalized scheme showing principal metal targets and protective mechanisms in yeast cells. See text for details.

case, and the effect is damaging to intact cells, then this should be manifest as an effect of metal-induced ROS on whole-cell metal toxicity. Many of the studies discussed below are consistent with this.

Evidence for a role of ROS in metal damage to yeasts includes increased metal-tolerance during anaerobicity (Greco *et al.,* 1990; Galiazzo *et al.,* 1991), protection exerted by certain free radical scavengers (Brennan and Schiestl, 1996), and the many overlaps in the molecular mechanisms used by yeasts to cope with oxidative and metal stress. Thus, overexpression of *OSR* (*ZRC1*) (which affects glutathione synthesis) increases yeast tolerance to lipid hydroperoxides, zinc, and cadmium (Kobayashi *et al.,* 1996), whereas deletion of the transcriptional regulator Yap1p (or homologue) results in H_2O_2 and cadmium hypersensitivity (Hirata *et al.,* 1994; Lesuisse and Labbe, 1995; Alarco and Raymond, 1999). Cadmium-dependent induction of Yap1 and Yap2 is also suppressible with the free radical scavenger N-acetyl-L-cysteine (Hirata *et al.,* 1994). The *ACE1* product, which regulates expression of

the metallothionein (*CUP1* and *CRS5*) and Cu,Zn-superoxide dismutase (*SOD1*) genes, is inducible with copper (Thiele, 1988; Gralla *et al.,* 1991; Strain and Culotta, 1996), while *SOD1* expression can determine sensitivity to both superoxide ($O_2^{\bullet -}$) (Gralla, 1997) and Cu (Greco *et al.,* 1990; Culotta *et al.,* 1995). *ACE1* deletion also renders *S. cerevisiae* hypersensitive to copper (Hu *et al.,* 1990). The type II thioredoxin peroxidase encoded by *AHP1* affects tolerance of both H_2O_2 and Mn^{2+} in *S. cerevisiae* (Farcasanu *et al.,* 1999). Furthermore, although metallochaperones do not function to protect cells from metal toxicity (O'Halloran and Culotta, 2000), they do have the potential to suppress oxygen toxicity. Thus, overproduction of Atx1p, which shuttles Cu to the intracellular Cu-transporting ATPase Ccc2p, protects cells against the toxicities of H_2O_2 and $O_2^{\bullet -}$ (Lin and Culotta, 1995). In addition, loss-of-function mutations in the transcriptional activator Mac1p, which is responsible for regulation of Cu homeostasis in *S. cerevisiae* at nontoxic Cu concentrations, results in hypersensitivity to Cd, Zn, Pb, and H_2O_2, whereas a gain-of-function mutation (*MAC1*^{up1}) confers a Cu-sensitive phenotype (Jungmann *et al.,* 1993b).

On first impression, the above evidence seems overwhelmingly in support of a role for ROS in metal toxicity. However, the complex interrelationships between the cellular biochemistry/homeostasis of ROS and metals in yeasts as well as other organisms are such that a closer appraisal is required. As discussed below, much of the evidence is ambiguous.

1. *The Role of Superoxide Dismutase*

Because the cytosolic superoxide dismutase, Sod1p, is the principal superoxide-scavenging enzyme of *S. cerevisiae* (Gralla, 1997), the well-documented influence of *SOD1* expression on cellular Cu sensitivity was considered a linchpin of the argument that ROS mediate Cu toxicity in yeasts. Indeed, the tight relationship between Sod1p function and metal toxicity/homeostasis in *S. cerevisiae* has been furthered by recent experiments indicating that superoxide toxicity in aerobic *sod1*Δ cultures may be mediated through enhanced iron-dependent oxidative damage (Corson *et al.,* 1999; Srinivasan *et al.,* 2000). However, with regard to Cu, it is now known that Sod1p has an alternative function (Table I). Culotta *et al.* (1995) presented several lines of robust evidence showing that the protection conferred by Sod1p against Cu is unrelated to the enzyme's superoxide scavenging activity. First, Sod1p enhanced Cu resistance under both aerobic and anaerobic conditions, which would seem to be inconsistent with a free radical-mediated effect (but see below). Moreover, genetic suppressors of the oxygen sensitivity of *sod1* mutants (*pmr1, bsd2, ATX1*) failed to suppress Cu sensitivity.

TABLE I

SOME KEY DETERMINANTS OF METAL RESISTANCE IN YEAST THAT ALSO EXHIBIT ANTIOXIDANT FUNCTION

Gene	Product	Principal metals shown to be detoxified	Principal activities
SOD1	Copper/zinc superoxide dismutase	Cu, Ag, Fe	Antioxidant; metal binding
GSH1	γ-Glutamylcysteine synthetase (rate-limiting for glutathione synthesis)	Cd	Antioxidant; metal binding
CUP1	Metallothionein	Cu, Cd	Metal binding; antioxidant
GLR1	Glutathione reductase	Se	Antioxidant; (metal binding)

Furthermore, Cu-induced *SOD1* transcription (via Ace1p) enhanced resistance to Cu but not ROS. It was concluded that Sod1p promotes Cu resistance through its capacity to bind and buffer cellular Cu (Culotta *et al.,* 1995). This conclusion was consistent with previous work indicating that Sod1p offered protection against silver, also via Ag binding (Cirilio *et al.,* 1994). Diminishment of metal availability could account for other reported instances of metal sensitivity in Sod-deficient organisms (Wisnicka *et al.,* 1998; Baysse *et al.,* 2000).

2. *The Effect of Anaerobicity*

There are conflicting reports of the influence of anaerobicity on copper toxicity in *S. cerevisiae*. Greco *et al.* (1990) found that many of the manifestations of Cu excess that lead to cell death were dependent on O_2. Enhanced Cu toxicity in aerobic cells compared to anaerobic cells of *S. cerevisiae* was also noted by Galiazzo *et al.* (1991). However, Strain and Culotta (1996) reported that Cu-dependent toxicity was greater under anaerobic conditions.

First, in the context of this review, it is important to stress that aerobicity/anaerobicity is a relatively blunt tool for seeking to correlate metal toxicity with ROS generation (such correlations were not the intentions of the above authors) since switching between the two conditions has broad consequences for yeast physiology. Greco *et al.* (1990) partly circumvented this problem by switching conditions just prior to short-term Cu exposure, before cells had the chance to adapt fully. Second, one manifestation of such a switch appears to be altered Cu uptake. Thus, anaerobic cultures of *S. cerevisiae* accumulate three- to tenfold more Cu than do aerobic cells during growth in Cu-supplemented medium

(Galiazzo *et al.,* 1991; Strain and Culotta, 1996). This effect probably accounts for the higher Cu toxicity during anaerobicity reported by Strain and Culotta (1996) and generally complicates interpretation of oxygen-(in)dependent Cu toxicity: Does oxygen availability directly influence the cells' abilities to tolerate Cu? Or is this effect a secondary consequence of altered Cu accumulation? In either case, that Cu causes toxicity at all under anaerobic conditions implies that mechanisms other than those dependent on ROS must contribute to Cu toxicity. A similar conclusion was reached by Dunning *et al.* (1998) during studies of Cu and Fe toxicity in bacteria. Indeed, anaerobic metal toxicity may be of particular relevance to yeasts such as *C. albicans,* which commonly occurs in the digestive tract of animals. Under such anaerobic (and acidic) conditions, the Cu-extrusion mechanism of *C. albicans* (Cacrp1p) becomes essential for survival in the presence of even very low copper concentrations (Weissman *et al.,* 2000). Although ROS can be eliminated as a likely cause of such toxicity, nonoxygen free radical species may still play a role. We have evidence that significant Cu-induced lipid peroxidation in *S. cerevisiae* may occur independently of mitochondrial ($O_2^{\bullet -}$-generating) function and in the presence of various $OH^{\bullet}$ scavengers (Avery lab, unpublished results). Such observations may be related to the propagation reactions of lipid peroxidation, which Cu can promote directly and independently of Fenton reaction catalysis, unlike lipid peroxidation initiation (Halliwell and Gutteridge, 1999). Dunning *et al.* (1998) also proposed that organic radical species, e.g., glutathione radicals, could mediate metal toxicity under anaerobic conditions. Thus, although anaerobicity provides a fairly effective means of uncoupling metal toxicity from oxidative stress, oxygen-independent mechanisms of free radical propagation might still potentiate metal damage under these conditions.

3. *The Role of Glutathione*

The protection conferred on yeasts against toxic metals by the antioxidant molecule glutathione (GSH) and GSH-dependent enzymes (Wu and Moye-Rowley, 1994; Wang and Oliver, 1997; Al-Lahham *et al.,* 1999; Pinson *et al.*, 2000), as well as the induction by cadmium of *YAP1* (Hirata *et al.,* 1994; Lesuisse and Labbe, 1995; Billard *et al.,* 1997) and *GSH1* (Stephen and Jamieson, 1997; Dormer *et al.,* 2000), is seemingly consistent with the toxicities of these metals being mediated by free radical-dependent mechanisms. However, like Sod1p, glutathione also binds metals, e.g., Cu(I) (Lin *et al.,* 1993) and Cd (Li *et al.,* 1997). Furthermore, in the case of arsenic, GSH is required to regenerate active Acr2p reductase that is required for the As detoxification pathway (Mukhopadhyay *et al.,* 2000). These interactions complicate

interpretation of the role of gluthathione in diminishing metal toxicity. Thus, is GSH-dependent metal resistance attributable to ROS scavenging by GSH, or to metal-GSH interactions that merely reduce the availability of metals for exerting toxicity? The latter explanation seems to apply to cadmium: it is now considered that GSH acts in a Cd detoxification pathway in *S. cerevisiae* that involves Ycf1p, a vacuolar GSH *S*-conjugate pump. Cd resistance may be determined by Ycf1-mediated cotransport of Cd with glutathione derivatives into the vacuole (Wemmie *et al.,* 1994; Li *et al.,* 1997). Therefore, while an understanding of the mechanism underlying GSH-dependent Cd resistance is of undoubted interest, in this case it has provided no new insight into the mechanism of Cd toxicity, i.e., it does not tell us what target(s) is/are protected as a consequence of GSH-dependent vacuolar Cd sequestration. Even so, it must be borne in mind that this action of GSH seems to be unique to Cd. It remains to be determined whether ROS scavenging by GSH accounts for the resistance to other metals that GSH confers and this warrants further investigation.

It should be noted that although the role of Yap1p in Cd resistance is generally attributed to control by Yap1p of *GSH1* and *YCF1* expression (Wemmie *et al.,* 1994; Wu and Moye-Rowley, 1994), Cd also elicits a Yap1/Yap2-dependent reduction in Fe uptake by *S. cerevisiae* (Lesuisse and Labbe, 1995). While such a mechanism could help compensate against Cd-dependent ROS generation (e.g., through diminished Fenton catalysis by Fe), Fe depletion in itself seems to contribute to the inhibition of *S. cerevisiae* by Cd (Lesuisse and Labbe, 1995).

4. *The Role of Metallothionein*

In common with Sod1p and glutathione, copper-binding metallothionein is also proposed to serve an antioxidant function in *S. cerevisiae* (Tamai *et al.,* 1993; Liu and Thiele, 1997). Thus, Cup1p can substitute for Sod1p *in vivo* (and *in vitro*) to protect against oxidative stress (Tamai *et al.,* 1993). The mechanism(s) of this protection by Cup1p has yet to be elucidated fully, but one component may be by diminishing the availability of uncomplexed Fe or Cu for catalyzing the Fenton reaction (Viarengo *et al.,* 2000). This example further underscores the difficulty of experimentally dissecting metal resistance mechanisms away from cellular antioxidant functions.

5. *Other Complicating Factors*

As mentioned earlier, the antioxidant properties of certain metals (e.g., Mn, Cu, Zn) add to the complexity of linking metal toxicity with free radical damage. In yeasts, metal protection against prooxidants can occur independently of Sod1p (so is not related to metal activation of

the enzyme) (Liu and Culotta, 1994), and is generally ascribed to ROS scavenging by free metal ions or metal complexes at nontoxic concentrations (Chang and Kosman, 1989; Liu and Culotta, 1994; Lin and Culotta, 1996). Such actions probably account for the suppression of the aerobic defects of *S. cerevisiae sod1* mutants by secondary mutations in *PMR1* (*BSD1*) and *BSD2* (Liu and Culotta, 1994; Lapinskas *et al.,* 1995; Liu *et al.,* 1997); *pmr1* or *bsd2* mutants accumulate higher levels of Mn and Cu (and Co and Cd), respectively, and also exhibit greater sensitivity to Mn and Cu when these metals are supplied at elevated concentrations. Bsd2p together with Mn normally promotes turnover of Smf1p and Smf2p, which are members of the Nramp family of metal transport proteins (Liu and Culotta, 1999a; Portnoy *et al.,* 2000). This posttranslational control minimizes the hyperaccumulation of toxic metals in wild-type cells and also provides a rapid switch for inducing metal uptake under conditions of metal starvation (Liu and Culotta, 1999b). Evidently, elevated metal accumulation in *bsd2* mutants can be beneficial where *S. cerevisiae* is impaired for enzymatic ROS scavenging.

Recent studies have also shown that, just as metals can affect cellular oxidant status, oxidants can alter metal metabolism and toxicity. Shinyashiki *et al.* (2000) demonstrated that nitric oxide regulates/disrupts metal homeostasis in *S. cerevisiae* via interactions with Ace1p. Although these interactions are manifested as an amelioration of Cu toxicity at low NO concentrations, Cu toxicity is greatly enhanced at high NO concentrations (Chiang *et al.,* 2000). The latter effect was attributed to inhibition by NO of Cu-inducible Ace1p activity and, therefore, seems to be determined ultimately by the usual Cu resistance activities (see above) rather than a direct exacerbation of Cu potency by NO.

6. *Nongenetic Heterogeneity in Cellular Metal Sensitivity*

The above evidence highlights how difficult it can be to delineate the mechanisms of metal toxicity solely from tests for altered metal sensitivity in organisms with manipulated genomes. Such tests should prove more informative in the context of metal toxicity if combined with other approaches. With regard to whole-cell studies, one recently introduced strategy for furthering our understanding of metal toxicity has been to explore phenotypic heterogeneity in yeast metal sensitivity, i.e., the variable metal susceptibilities of individual cells within yeast cultures. Differential metal sensitivity within isogenic yeast cultures is observed frequently, although until recently there had been no serious attempt to provide an explanation for it. A principal variable among cells in exponential microbial cultures is cell cycle stage. Using flow

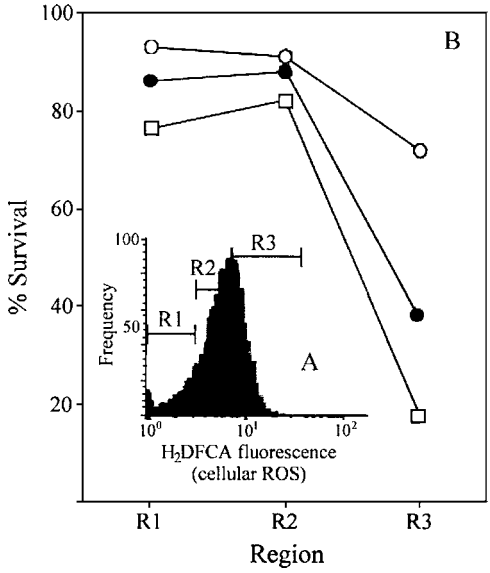

FIG. 4. Influence of initial oxidant status and PUFA-loading on Cu sensitivity of *S. cerevisiae,* measured with flow cytometry. (A) Cells stained with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFA) were sorted on the basis of low (R1), medium (R2), and high (R3) initial oxidant status. (B) Each sorted sub population (R1–R3) was exposed to $Cu(NO_3)_2$ and the number of viable cells determined by staining with propidium iodide. Percentage viabilities are shown for regions 1, 2 and 3 of unsupplemented (○), linoleate- (●), and linolenate- (□) supplemented cultures. Adapted from Howlett and Avery (1999), with permission from Elsevier Science.

cytometry, Howlett and Avery (1999) showed that variable Cu sensitivity in exponential phase *S. cerevisiae* is cell cycle dependent and, therefore, is not a stochastic phenomenon. G_1/S-phase cells of *S. cerevisiae* tended to be more resistant to Cu than G_2/M-phase cells. Moreover, this dependence on cell cycle stage was linked to the initial oxidant status of the cells. Thus, cells that exhibited the highest fluorescence with the ROS-sensitive probe 2′,7′-dicholorodihydro-fluorescein diacetate prior to Cu exposure, and/or cells previously enriched with polyunsaturated fatty acids (which enhance susceptibility to lipid peroxidation, see Sect. VI.B), were the most sensitive to Cu (Fig. 4). This evidence strongly suggests a role for oxidative stress in determining heterogeneous Cu sensitivity.

Recent evidence also suggests a link between the cadmium and ROS sensitivities of individual cells in *S. cerevisiae* cultures. Wang *et al.* (2000) showed that cellular Cd, H_2O_2, and menadione resistance all oscillated in synchrony with ultradian rhythms in continuous

S. cerevisiae cultures; these rhythms are usually coupled to the cell cycle. However, there was a slight (~60°) phase difference in the peaks of resistance to Cd and the prooxidants (Wang *et al.,* 2000), suggesting that there is some facet to Cd toxicity other than can be described solely by H_2O_2 or $O_2^{\bullet -}$.

Molecular bases for these correlations between the metal and ROS sensitivities of individual cells have not been elucidated. That fluctuations in Cu and Cd sensitivity occur over relatively short time scales (*S. cerevisiae* cell cycle time, ~2 h) seems to be in keeping with the dynamic regulation of metalloregulatory transcription factors such as Mac1p and Ace1p and the products that they regulate, such as Cup1p (Pena *et al.,* 1998; Mateus and Avery, 2000). Although microarray data indicate that that these proteins are not subject to cell cycle dependent transcriptional regulation (Spellman *et al.,* 1998), cell cycle control at the posttranscriptional level remains a possibility. Alternatively, cell cycle dependent metal sensitivity may be more closely related to a broader physical property—such as susceptibility of cellular macromolecules to oxidative damage—than to the expression of specific genetic determinants. The evidence from heterogeneity studies to date is consistent with a role for ROS in the toxicities of Cu and Cd.

B. Metal-Induced Membrane Damage

One of the most widely observed effects of metal toxicity toward yeasts and other organisms is a sudden increase in membrane permeability, which is generally manifest as a rapid loss of intracellular ion pools (e.g., that of K^+) or enhanced accumulation of extracellular ions (e.g., H^+ and Ca^{2+}) (Gadd and Mowll, 1983; Kessels *et al.,* 1987; White and Gadd, 1987a; Ohsumi *et al.,* 1988; Avery *et al.,* 1996). Membrane perturbation is considered a key potential mechanism of metal toxicity (Gadd, 1993). Although effects such as cation efflux have been commonly used as indices of metal toxicity, the precise mechanisms underlying loss of microbial membrane integrity are still poorly understood.

In addition to membrane permeabilization, metal toxicity at the plasma membrane may be manifest in subtler ways, such as through effects on the activities of membrane-bound enzymes. It is well known that many integral membrane proteins are markedly dependent on their membrane–lipid environments for optimal activity and that membrane perturbations generally elicit diminished function (In't Veld *et al.,* 1993). Thus, metal-dependent inhibition of plasma membrane H^+-efflux activity is readily measurable in *S. cerevisiae* and, like cation leakage, has been used as a physiological indicator of metal (and organometal) toxicity (White and Gadd, 1987b;

Karamushka *et al.,* 1996; Hoptroff *et al.,* 1997; Masia *et al.,* 1998; Karamushka and Gadd, 1999). Copper-dependent inhibition of the *S. cerevisiae* plasma membrane H^+-ATPase occurs at concentrations in excess of 1.5 m*M* Cu (Fernandes *et al.,* 1998). Lesser perturbation of plasma membrane integrity during mild Cu stress seems to favor H^+-ATPase activation. Plasma membrane H^+-ATPase activity may actually contribute to Cu tolerance in *S. cerevisiae* (Fernandes and Sa-Correia, 1999; Fernandes *et al.,* 2000). The perturbation of H^+-ATPase activity at high Cu concentrations was suggested to arise from disorganization and fluidization of the plasma membrane (Fernandes *et al.,* 1998). Cd-, Cr(III)-, and Cu-induced plasma membrane "fluidization" has been demonstrated in *S. cerevisiae* with fluorescence depolarization and electron spin resonance techniques (Assmann *et al.,* 1996; Howlett and Avery, 1997a; Fernandes *et al.,* 2000; Pesti *et al.,* 2000). But what is the mechanism(s) that underlies such effects of metals on membrane physical properties?

The first clue to answering this question comes from the fact that metabolic activity is usually a prerequisite for metal-induced membrane leakage (Ohsumi *et al.,* 1988; Avery *et al.,* 1996), which indicates that a physical metal-binding process cannot be the direct cause. It is also known that, as in higher organisms, many metals promote lipid peroxidation in yeasts (Lee *et al.,* 1996; Howlett and Avery, 1997a; Mannazzu *et al.,* 2000). However, to demonstrate metal-induced lipid peroxidation is one challenge. To demonstrate that this is the cause of metal-induced membrane damage is considerably more difficult. The time scales of metal-dependent lipid peroxidation and membrane damage may be correlated (Howlett and Avery, 1997a), but such evidence is indirect particularly since indices used for measuring membrane physical properties, permeabilization, and lipid peroxidation are unlikely to be linearly related. Even different methods for measuring lipid peroxidation can give quite different results (Howlett and Avery, 1997a). A more meaningful approach to relating gross membrane damage to lipid peroxidation is specifically to manipulate susceptibility to lipid peroxidation and then to test for corresponding changes in susceptibility to gross membrane damage. We have compared organisms either enriched for or lacking polyunsaturated fatty acids (PUFAs). The oxidizability of PUFAs means that PUFA-rich membranes are more susceptible to lipid peroxidation than are membranes that comprise predominantly saturated or monounsaturated fatty acids (Dix and Aikens, 1993). *Saccharomyces cerevisiae* readily incorporates exogenous PUFAs to greater than 60% of total membrane fatty acids, with no deleterious effects on growth under nonstress conditions (Avery *et al.,* 1996; Howlett

and Avery, 1997b). However, PUFA-rich yeasts exhibit a marked sensitivity to Cu- and Cd-induced plasma membrane permeabilization and whole cell toxicity (Avery *et al.,* 1996; Howlett and Avery, 1997a,b). Furthermore, these effects are correlated closely with elevated levels of metal-induced lipid peroxidation in PUFA-rich cells (Howlett and Avery, 1997a). These findings suggest that lipid peroxidation is the cause of elevated metal sensitivity in PUFA-rich *S. cerevisiae* and complements reports of high background levels of lipid peroxidation in metal-exposed PUFA-rich higher systems (De Vos *et al.,* 1993; Furuno *et al.,* 1996), although in those cases relationships with whole-cell damage were not sought.

It should be stressed that as well as enhancing membrane susceptibility to oxidation, PUFA enrichment also alters membrane physical properties such as fluidity, which in themselves could conceivably influence metal sensitivity. *Candida albicans erg*$^{-}$*2* mutants that are defective for ergosterol synthesis were found recently to exhibit greater sensitivity to Cr(III) than wild type *C. albicans,* which was correlated with a higher membrane rigidity of the mutant (Pesti *et al.,* 2000). However, PUFA supplementation—which also enhances metal sensitivity—diminishes membrane rigidity (Howlett and Avery, 1997a), thereby undermining a simple correlation between rigidity and metal sensitivity. Furthermore, as well as affecting ergosterol content, the *erg*$^{-}$*2* mutation elicits an increased content of unsaturated fatty acids in *C. albicans* (Pesti *et al.,* 2000), and consistent with a role for lipid peroxidation, this could be the true cause of the Cr(III) sensitivity of these cells.

It has also been suggested that metal-induced alterations in membrane physical properties could be an indirect mechanism by which metals enhance lipid peroxidation (Verstraeten and Oteiza, 1995). However, studies in our laboratory were inconsistent with this since metal-induced lipid peroxidation preceded any discernible effects on membrane order (Howlett and Avery, 1997a).

In our studies, Cu was more toxic at the whole-cell level than Cd, yet Cd generated the greater extent of plasma membrane permeabilization, suggesting that membrane permeabilization may make a more important contribution to Cd toxicity than to Cu toxicity in *S. cerevisiae*. This could be related to the differing pathways of lipid peroxidation that these metals seemed to exacerbate (Howlett and Avery, 1997a). Similar comparisons between Cu and Cd have been made in studies with higher eukaryotic systems (Pourahmad and O'Brien, 2000). Here, the relative effects of the two metals on mitochondrial membranes were more closely correlated with various measurements of oxidative stress than were their relative effects on plasma membranes. It was concluded that Cu-induced oxidative damage occurred as a result of mitochondrial

ROS formation, independent of cytosolic ROS formation due to redox cycling (Pourahmad and O'Brien, 2000).

It should be noted that although PUFA enrichment caused *S. cerevisiae* to be sensitized to Cu and Cd, the reverse was true with the organometal tributyltin chloride (Masia *et al.,* 1998). This was unexpected since the reactivity of available radicals with organotin carbon–metal bonds is considered a means by which organometals may generate membrane damage (Mehlhorn, 1986). The mechanism underlying the tributyltin resistance of PUFA-enriched *S. cerevisiae* has yet to be elucidated, but effects of fatty acid composition on organometal solubility in biological membranes could play a role (Masia *et al.,* 1998). Such effects could mask any enhancement of tributyltin-dependent lipid peroxidation in PUFA-rich cells.

Overall, the evidence to date indicates that lipid peroxidation may well account for the membrane permeabilization evident during Cu and Cd (and possibly Cr) exposure of yeasts. This membrane damage appears to be a major factor in the killing of PUFA-rich yeasts during metal exposure. However, it may not be so important in cells comprised predominantly of saturated and monounsaturated fatty acids, which are markedly less susceptible to lipid peroxidation. Yeasts such as *Candida* spp. synthesize PUFAs naturally whereas *S. cerevisiae* does not, and this could have implications for the relative importance of membrane damage in the metal susceptibilities of these organisms.

C. Metal-Induced Protein Damage

Metal-induced protein damage generally has been less well studied than the effects of metals on biological membranes. However, protein targeting by metals has received increased attention recently, which may partly reflect improvements in methods of detection and analysis. Jungmann *et al.* (1993a) reported that cadmium activates ubiquitin-dependent proteolysis in *S. cerevisiae* and that proteolysis-deficient mutants are hypersensitive to Cd. This indicated that Cd-induced formation of abnormal proteins could be a major reason for Cd toxicity, particularly if such proteins are not efficiently degraded. There have been several reports of specific protein activities that are inhibited by metal stress. Glutathione reductase from yeast is markedly inhibited by Cu, which has been proposed as an indirect mechanism by which Cu might promote oxidative stress, i.e., via depletion of reduced glutathione (Murakami and Yoshino, 1999). Metal-induced glutathione depletion is known to occur in *S. cerevisiae* (Fortuniak *et al.,* 1996) and in higher eukaryotes (Stohs and Bagchi, 1995). However, as is commonly the case, causal links between these effects and the toxicology of metals have not been

established. Thus, with regard to enzymes, it can be assumed that almost any activity will be inhibited given a sufficiently high metal concentration, but specific enzymes or other targets may be far more pertinent to metal toxicity *in vivo* (e.g., vital cellular constituents that are inhibited or perturbed at comparatively low metal concentrations). This point was illustrated in an excellent recent study by Naganuma *et al.* (2000), in which L-glutamine:D-fructose-6-phosphate amidotransferase (GFAT) was specifically identified as a key target of methylmercury (MeHg) toxicity in *S. cerevisiae*. GFAT is an essential enzyme that catalyses the synthesis of glucosamine-6-phosphate and is conserved in a wide range of organisms including *E. coli,* mice, rats, and humans. The toxicity of MeHg was suppressed by overexpression of *GFA1,* which encodes GFAT, or by loading excess glucosamine-6-phosphate into *S. cerevisiae* (Naganuma *et al.,* 2000). Furthermore, GFAT activity was inhibited by 90% at MeHg concentrations that gave almost no inhibition of other sulfhydryl-enzymes such as alcohol dehydrogenase, glutathione reductase, and lactate dehydrogenase. It was proposed that MeHg might bind to Cys-2 at the amino-terminal end of GFAT, thereby diminishing GFAT activity. However, this hypothesis was not tested experimentally, which leaves the door open for other possible explanations. One such explanation could be that GFAT is susceptible to metal-induced oxidative damage, as may be the case for many metal-sensitive proteins. There have been no direct studies of metal-induced oxidation of specific proteins in yeasts, but this can now be done more readily with the availability of antibodies against derivatized carbonyl groups on oxidatively modified proteins (Avery *et al.,* 2000; Cabiscol *et al.,* 2000). This approach has been used recently to detect $H_2O_2^-$ and $O_2^{\bullet -}$-sensitive proteins in *S. cerevisiae* (Cabiscol *et al.,* 2000). GFAT was not among the several specific glucose-metabolic enzymes that were identified by these workers, but their scope was limited to proteins from whole-cell extracts that were discernible in one-dimensional gels.

D. Metal-Induced DNA Damage

There have been few studies of metal-induced DNA damage in yeasts. This is surprising considering the extent of work on other aspects of yeast–metal interactions together with the detailed understanding of DNA damage repair systems in *S. cerevisiae* (see Sect. IV), and needs to be addressed. Metals such as cadmium, lead, and selenium have been shown to promote various types of DNA damage in *S. cerevisiae,* including mutation, recombination, and telomere-length alterations (Brennan and Schiestl, 1996; Cheng *et al.,* 1998; Yuan and Tang, 1999; Cui and Tang, 2000). It has been proposed that selenite-induced DNA damage

promotes *RAD9*-dependent cell cycle arrest in *S. cerevisiae* (Pinson *et al.,* 2000), which may account for some of the growth-inhibitory effects of Se. Using *SUP*-based mutation assays, Cheng *et al.* (1998) presented two lines of evidence consistent with Cr(VI)-induced DNA damage being ROS dependent: first, mutagenesis was enhanced in yeast cells lacking Apn1p, an enzyme with the capacity to repair oxidative (among other) DNA lesions; second, the patterns of mutation in Cr(VI)-treated mammalian cells were similar to those generated by ionizing radiation or hydrogen peroxide. Evidence in support of a role for ROS in Cd-dependent DNA damage and toxicity in *S. cerevisiae* was presented by Brennan and Schiestl (1996). Cd-induced DNA recombination and toxicity were suppressed by the antioxidant molecule N-acetylcysteine and by anaerobicity. In addition, Cd activated an intracellular free radical-sensitive reporter compound and Cd hypersensitivity was evident in *sod1* and *gsh1* mutants (Brennan and Schiestl, 1996). Certain of these results could potentially be attributable to free radical-independent mechanisms (see Sect. VI.A). Even so, the weight of evidence from studies to date is strongly in keeping with DNA damage being an important mechanism of metal toxicity and with that damage being ROS mediated, at least in the cases of Cd and Cr(VI).

VII. Genomic Approaches to Understanding Metal Toxicity

As discussed in Section VI.A, studies with organisms that have been manipulated to overexpress one or two genes or to harbor relevant deletions have not yielded significant insight into the mechanism underlying metal toxicity; where diminished metal toxicity in a modified strain proves to result from altered metal homeostasis and/or availability (as is the case in most examples), the question that remains is, What is the mechanism of toxicity that is affected by altered metal availability? One obvious way to address this problem would be to focus on products that, unlike Sod1p and glutathione, do not have the potential to influence metal availability. Appropriate selection of such candidates for study is complicated by the facts that many characterized proteins do bind metals and that the metal binding capacities of uncharacterized cellular proteins are unknown. These are particular problems where choice is limited to proteins that also influence metal sensitivity.

An alternative approach to studying functions and phenotypes associated with single gene products is to cast the net wider through genome-wide strategies. Recent functional genomics technologies are ideally suited to the elucidation of gene products required for tolerance or resistance to metal stress, and are beginning to be exploited in this way.

Yeast DNA microarrays enable rapid screening for genes that are induced under different physiological conditions, e.g., metal stress vs no stress. Since the anticipated functions of many such genes induced during metal stress would be in cellular protection against metal toxicity, their identification has considerable potential for providing insight to metal action. Gross *et al.* (2000) recently used microarray technology to screen for genes induced by copper stress in *S. cerevisiae*. Five transcripts exhibiting increased abundance were identified in cells treated with 100 μM $CuSO_4$. The observed induction of *SOD1* and metallothionein-encoding *CUP1* and *CRS5* transcripts was consistent with previous studies, which established Ace1p as the transcription factor through which these responses are mediated (Thiele, 1988; Gralla *et al.,* 1991; Strain and Culotta, 1996). The value of the microarray approach was reflected in the identification of two genes not previously known to be induced by Cu: *FET3* and *FTR1*. Results indicated that a Cu-dependent transient diminution of cellular iron led to the induction of the latter genes, which function in high affinity iron uptake (Gross *et al.,* 2000). An effect of Cu on cellular Fe status represents a stress additional to that which can be attributed directly to Cu and is reminiscent of similar effects during Cd stress (Lesuisse and Labbe, 1995).

Bell *et al.* (1999) used flow cytometry to enrich for copper-inducible clones from a green fluorescent protein-based promoter library in *S. cerevisiae*. After repeated selection (by sorting) for clones exhibiting Cu-induced EGFP expression, one sequence of interest was identified. The sequence harbored a putative Ace1p binding site, consistent with induction by Cu, and putative Yap1p and Hap2/3/4p sites in divergent sequences (Bell *et al.,* 1999). The promoter of interest occurred between two ORFs of unknown function (*YFL055W* and *YFL054C*). However, these ORFs are members of membrane-transport protein families and further elucidation of their potential roles in the copper stress response is warranted.

Although a detailed overview of metal uptake/distribution in yeasts is beyond the scope of this review, it is worth mentioning that DNA microarrays may also be used to find genes involved in metal homeostasis. Lyons *et al.* (2000) compared expression profiles of zinc-deficient, zinc-sufficient, and *zap1*-mutant *S. cerevisiae* (Zap1p is a transcriptional activator involved in zinc homeostasis). With this approach, forty-six genes potentially subject to Zap1p regulation were identified, and regulation by Zap1p was confirmed using *lacZ* reporter fusions for at least seven of the newly identified target genes (Lyons *et al.,* 2000).

Thus, microarrays can provide valuable new information on cellular responses to metal stress and the regulatory pathways involved. However, the results are limited to transcriptional-level responses. In

contrast, cellular protein profiles provide information that should be more closely related to cell function since these additionally encompass the influence of posttranscriptional and degradation regulatory mechanisms (albeit not posttranslational modifications with any precision). One drawback of proteome analyses is that with current technologies—usually two-dimensional (2D) gel electrophoresis—at best only approximately one quarter of yeast proteins are resolvable (Gygi *et al.,* 2000). Nonetheless, recent technical developments enabling better separation, reproducibility, and protein identification have coincided with studies involving proteome analyses applied to metal-stressed yeasts. Using 2D gel electrophoresis, Mannazzu *et al.* (2000) showed that vanadate and copper caused significant increases in the levels of many antioxidant enzymes in *Hansenula polymorpha*. There were differences between the metals. For example, the extent of catalase induction by vanadate was far greater than by Cu, yet vanadate did not cause lipid peroxidation in *H. polymorpha* whereas Cu did. This supported physiological evidence that, like Cu and Cd (Howlett and Avery, 1997a), Cu and V may promote differing mechanisms of oxidative stress and that such stress could govern their toxicities (Mannazzu *et al.,* 2000).

As discussed earlier (Sect. VI.A), the Yap1p transcriptional regulator of *S. cerevisiae* affects Cd and H_2O_2 sensitivity in tandem, as is also the case for Cap1p of *C. albicans,* which is similar structurally and functionally to Yap1p (Alarco and Raymond, 1999). Such evidence is in keeping with these stressors exerting toxicity by similar mechanisms. However, a hyperactive truncated allele of *CAP1* confers Cd but not H_2O_2 resistance (Alarco and Raymond, 1999), and another transcriptional regulator of *S. cerevisiae,* Skn7p, is important for H_2O_2 but not Cd resistance (Lee *et al.,* 1999). The discrepancy between the phenotypes of *yap1* and *skn7* mutants exposes a clear difference in the actions of Cd and H_2O_2, which provides an opportunity to determine more precisely those genes of the Yap1 regulon that may be responsible for conferring Cd resistance, i.e., discount those that are also under Skn7 control. Lee *et al.* (1999) took a major step in this direction by using 2D gel electrophoresis to compare the Yap1 and Skn7 regulons. An Skn7-independent subset of the Yap1-regulon was identified that comprised several activities of the glutathione pathway, e.g., Cys3p, Gsh1p, Glr1p. Several genes of the pentose phosphate pathway were also identified, which could be important in electron-supply to the glutathione pathway via NADPH (Lee *et al.,* 1999). A role for GSH in yeast cadmium resistance was already known, as were the potential mechanisms underlying GSH-dependent metal resistance (see Sect. VI.A.3). However, this work further illustrates the potential power of genome-wide approaches for giving new insight to metal toxicity. Such insight is bound

to be furthered in the coming years as genomic/proteomic applications become more commonplace.

VIII. Conclusions and Future Directions

Metal toxicity continues to be a major problem at several levels, and there is a pressing need for the deleterious effects of metals at the cellular and molecular level to be understood. Most of the evidence discussed in this review is consistent with a major role for free radical generation in the toxicities of many metals. A number of mechanisms have been proposed to underlie metal-induced ROS generation, and these mechanisms tend to differ between metals according principally to whether they are redox active or inactive. Redox-active metals such as Cu, Cr, Fe, V may directly promote free radical formation in cells (e.g., by Fenton catalysis), whereas indirect effects (e.g., glutathione depletion) may account for elevated free radical damage during exposure to nonredox active metals such as Cd and Hg. While free radical/ROS-independent mechanisms of toxicity have also been suggested for certain metals in yeasts and other organisms, few of these alternatives have been tested or proven. At the same time and as emphasized above, certain of the evidence that ROS generation mediates metal toxicity is indirect and open to alternative interpretation. Such concerns cannot be attributed to limitations of the experimental system in the case of *S. cerevisiae*.

The interactions of yeasts with toxic metals are of considerable interest not only because of the commercial and medical importance of these organisms, but also because they serve as excellent eukaryotic models for delineating the mechanism(s) of metal toxicity (as well as oxidative stress responses and metal homeostasis). Several yeast groups are currently focusing on metal homeostasis, which may have steered some attention away from exploiting yeasts for direct studies of metal toxicity. Moreover, there is a strong emphasis on molecular genetics in much recent yeast research, yet molecular genetics alone has failed to further significantly our understanding of the mechanism(s) of metal action (see above), at least in the absence of simultaneous measurements of metal damage. Results derived from manipulation of cellular metallothionein, superoxide-dismutase, and glutathione levels can be ambiguous since these determinants have more than one potential mode of action: metal binding and antioxidant. What is needed now is a marriage of genetic, biochemical, and physiological approaches applied to this problem; *S. cerevisiae* is highly amenable to all of these. It is the few studies that have implemented such broad strategies recently that have provided the greatest insight into metal toxicity.

The evidence to date indicates that each of the major cellular macromolecules (lipids, proteins, nucleic acid) can be a target for metal toxicity. The extent of the contribution to whole-cell metal toxicity made by damage to each potential target has not been determined, and this is not helped by the fact that almost all studies have tended to focus on one type of target and/or whole cells exclusively. Furthermore, the principal target as well as the nature of the damage to that target can depend on the metal. Nonetheless, there is now strong evidence that oxidative processes probably account for the bulk of the damage to DNA and membranes caused by at least some metals in yeasts. The relative importance of membrane damage in determining the metal sensitivities of different yeasts may depend on the organisms' lipid compositions, specifically their polyunsaturated fatty acid contents. Specific protein targets of metal toxicity have also been identified, but it remains to be seen whether metal-induced protein oxidation is the cause. Novel genome-wide screening technologies have recently helped in the characterization of new genes that are induced in response to metal stress, and such approaches hold great promise for providing further insight in the coming years. In addition to the relevance to human health, such advances will be pertinent to understanding the fitness of yeasts and fungi in industrial (and possibly laboratory) settings and their potential control where proliferation is undesirable.

Acknowledgment

The financial support of the National Institutes of Health (R01 GM57945) for the author's work on metal toxicity is gratefully acknowledged.

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Foodborne Microbial Pathogens and the Food Research Institute

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I. Introduction

It is surely apparent to anyone who follows the news that microbiological food safety has come of age. In fact, one might say, "... has *finally* come of age," because not long ago the news media seemed mostly interested in the safety of chemical food additives. That has changed, however, and foodborne illness is now widely recognized as an important public health issue.

In the early days of research on microbiological aspects of food safety, investigations were focused on basic questions such as the following: What pathogen caused this outbreak of foodborne disease? How much salt do we need to add to a food to prevent pathogen growth? What

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0065-2164/01 $35.00

processing conditions are necessary to produce canned food without botulism? Some chemical contaminants, e.g., lead, and certain poisonous plants and animals, were recognized as causes of illness associated with foods but relatively few microbes were known to be important causes of foodborne disease.

In recent years, food safety experts have had to contend with many more recognized microbial pathogens and have come to realize that virulence in bacteria varies in different strains. Moreover, virulence characteristics of specific strains are not always constant as microbes adapt to their environment and acquire new genetic information from other organisms. Research on methods for safe food processing and preparation has provided evidence that microbes subjected to some stresses such as high salt concentrations often become more resistant to other limiting factors such as high temperatures.

In this review, we briefly trace developments in the microbiology of food safety during the past 55 years as exemplified by research activities at the Food Research Institute (FRI), first in Chicago and then at the University of Wisconsin. Then we discuss issues that may lie ahead with respect to newly emerging pathogens and new challenges to food safety caused by globalization of the food supply, aging of the population, changes in plant and animal agriculture, and changes in consumer preferences for different foods. To cope with food safety concerns in the present and future, we should take a more holistic view: (1) Production of safe foods should be considered an ongoing process from the origins of meat and vegetables on farms through preparation, processing, storage, and serving at home or in a restaurant or cafeteria. (2) Pathogenic bacteria must be understood in the context of their total environment. Not only do physical factors (heat, water activity, atmosphere) affect growth and toxicity of these bacteria, but interactions with other microbes in the environment may inhibit the growth of pathogens, or conversely, may enhance their harmful effects. As we learn more about these processes, we should be better able to cope with present food safety problems and to anticipate and prevent future problems.

II. Historical Overview

The emergence of microbes as an important cause of foodborne illness is mirrored by the changing emphasis on food safety research conducted by FRI. FRI was founded at the University of Chicago in 1946 by Dr. Gail Dack with the support of a number of food industry sponsors who foresaw the potential for food safety problems as food manufacturing expanded after the war. When Dr. Dack retired in 1966, FRI moved to the University of Wisconsin, and under the leadership

TABLE I

CHRONOLOGY OF MAJOR FOODBORNE PATHOGENS UNDER STUDY AT FRI

Years studied	Organisms and toxins
1946–Present	*Clostridium botulinum, Salmonella, Staphylococcus aureus*
1960s–Present	*Clostridium perfringens,* viruses, toxigenic molds (mycotoxins), algal toxins
1970s–Present	*Bacillus cereus*
1980s–Present	*Listeria monocytogenes, Campylobacter jejuni, Escherichia coli* O157:H7

of Dr. Mike Foster, expanded to include more faculty and more varied research projects. Today the FRI team numbers nearly 80 faculty, pre- and postdoctoral students, scientists, researchers, and laboratory and office support personnel. Collaboration in the institute's multidisciplinary basic and applied research program involves scientists from many other departments in the university. Some 40 research projects are in progress at any given time, some involving basic research while others reflect immediate needs and new priorities within the food industry. Research activities are supported by grants from the food industry, federal and state competitive grants, and sponsor contributions.

Since its inception, research at FRI has focused primarily on the microbiology of foodborne disease (Table I). For the first 25 years or so, three pathogens were of major concern: *Clostridium botulinum, Salmonella* spp., and *Staphylococcus aureus.* Of these, *S. aureus* was the leading known cause of food poisoning with 77 outbreaks and 3798 cases reported in 1952 compared to 2 outbreaks and 5 cases for *C. botulinum* and 42 outbreaks and 1491 cases of salmonellosis. Many of the *Salmonella* spp. outbreaks were traced to contaminated water, not food, and about one fourth of the outbreaks were of typhoid fever due to *S. typhi* (Dauer, 1961).

A. *STAPHYLOCOCCUS AUREUS*

As early as 1914, staphylococci in milk were known to produce a toxin which caused illness (Barber, 1914). This was later confirmed by Dr. Dack, the first director of FRI, and some of his students who intentionally sampled some contaminated cake and then cell-free filtrates of *S. aureus* cultures (Dack *et al.,* 1930). One of the first research groups at FRI, led by Dr. Merlin Bergdoll, investigated the nature of the toxins produced by *S. aureus* and devised methods for their detection. Over a period of many years, seven enterotoxins were identified, purified,

and characterized: A (Chu *et al.,* 1996), B (Bergdoll *et al.,* 1959; Lopes *et al.,* 1996), C_1 (Borja and Bergdoll, 1967), C_2 (Avena and Bergdoll, 1967; Lopes *et al.,* 1996), C_3 (Reiser *et al.,* 1984), D (Chang and Bergdoll, 1979), and E (Bergdoll *et al.,* 1971; Borja *et al.,* 1972). Antibodies to these toxins were produced, and radioimmunoassays and enzyme-linked immunoassays were developed for the detection of enterotoxins in foods (Robbins and Bergdoll, 1984; Miller *et al.,* 1978; Freed *et al.,* 1982). Other research, conducted by Dr. H. Sugiyama and colleagues, concerned the mode of action of the staphylococcal enterotoxins (Sugiyama *et al.,* 1961; Sugiyama and Hayama, 1965). Yet another enterotoxin, H, was recently identified, isolated, and characterized (Pereira *et al.,* 1996; Su and Wong, 1995, 1998). Many other researchers in other labs have contributed to our knowledge of these toxins and have devised effective methods for their detection in foods (Balaban and Rasooly, 2000).

In the 1980s, *S. aureus* was linked to toxic shock syndrome and FRI researchers investigated the nature and production of toxic shock syndrome toxin by these bacteria (Bergdoll and Chesney, 1991; Wong and Bergdoll, 1990). *Staphylococcus aureus* continues to be an important foodborne pathogen, with outbreaks most often associated with ham (Ward *et al.,* 1997). However, the relative importance of this pathogen has declined as other foodborne bacteria have been identified and have become more prominent.

B. *Salmonella* spp.

Many serotypes of *Salmonella* have been recognized as human pathogens. Early in the past century, typhoid fever (*S. typhi*) was a fairly common form of salmonellosis in the United States. With improvements in sanitation and drinking water purification, the incidence of this disease has decreased in the United States from thousands of cases/year in 1940 and 1950 to 552 reported cases in 1990. Most of the cases of typhoid fever now diagnosed in the United States were acquired abroad. However, the reported prevalence of nontyphoid salmonellae has increased from a few thousand cases in the early 1950s to over 45,000 cases in 1996 (Centers for Disease Control, 1990, 1996a). It has been estimated that the total number of cases in the United States now is close to 1,500,000 per year (Mead *et al.,* 1999). Over the years, many foods have been implicated as vectors for salmonellosis (Foster, 1989). During the 1940s and 1950s, several outbreaks of salmonellosis were traced to dried eggs, yeast, and coconut. Later, researchers at FRI and elsewhere investigated outbreaks and growth of salmonellae in milk (Marth, 1969),

cheese (Johnson *et al.,* 1990), chocolate (Goepfert and Biggie 1968), and other processed foods.

Numerous serotypes of *Salmonella* have been associated with foodborne outbreaks. Although the actors have changed somewhat in the past two decades, salmonellosis continues to be a major foodborne illness. *Salmonella enteritidis* rose from a relatively rare isolate in the 1960s to a major pathogen associated with eggs in the 1980s (Bäumler *et al.,* 2000). By 1988, 15,427 human cases of *S. enteritidis* were reported in England and Wales as compared to 6444 cases of *S. typhimurium* which was previously the most common isolate. A similar rapid rise in cases was observed in the United States. In 1997 the epidemic appeared to peak with 7924 cases of *S. enteritidis* reported by CDC in the United States and 23,008 reported by the Public Health Laboratory Service in England.

A dramatic increase in *S. typhimurium* isolates which are resistant to 5 or more antibiotics has also been reported since 1990. As many as 65% of all *Salmonella* spp. cultured from cattle in the United Kingdom have been identified as a strain of *S. typhimurium* DT104, resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline. Some strains were also resistant to trimethoprim, spectinomycin, and/or ciprofloxacin (Low *et al.,* 1996; Threlfall *et al.,* 1996). Antibiotic resistance has become an increasing problem in a number of medically important bacteria.

C. *Clostridium botulinum*

Although the number of outbreaks and cases of botulism are relatively low compared to some other types of foodborne illness, the high fatality rate of this disease has made it an important topic of ongoing research. Some of the early FRI researchers had previous experience during World War II in the U.S. Army labs where they grew cultures of *C. botulinum* and worked to purify its toxins. After the war, research at these biological warfare labs was cut back and these scientists used their experience to help solve the problems posed by foodborne botulism. By this time safe canning procedures had been developed so that cases related to commercially canned foods were rare. Yet *C. botulinum* continued to be problematic for home canners and also appeared in some unexpected foods—for example, Type E botulism in vacuum-packaged Great Lakes fish (Foster, 1989) and *C. botulinum* in fresh mushrooms packaged in plastic wrap (Sugiyama and Yang, 1975).

Microbiological studies of the factors promoting and limiting the growth of *C. botulinum* were undertaken by Dr. Foster and later

Dr. Sugiyama and their colleagues (Sugiyama *et al.,* 1972). In 1975, infant botulism was recognized as a problem and Dr. Sugiyama's lab investigated this illness using a mouse model (Sugiyama and Mills, 1978). Later Dr. Eric Johnson and colleagues continued research on botulism in various foods and also investigated the genetics of botulinum toxin production (Johnson *et al.,* 1997; Lin and Johnson, 1995; Zhou *et al.,* 1995). Current research aims at improving methods for toxin detection and determining the structure of a clostridial bacteriocin and its effects on *C. botulinum.*

At the same time, intensive research on the chemical structure of botulinum toxin was being conducted in Dr. DasGupta's lab. Botulinum neurotoxins were isolated from cell cultures, and their subunit structures and amino acid compositions were determined (DasGupta and Sugiyama, 1972; DasGupta and Sathyamoorthy, 1984; Schmidt *et al.,* 1984). Further research revealed the importance of zinc in the structure and function of the toxin (Schiavo *et al.,* 1992), and finally the complete three-dimensional crystal structure of the botulinum neurotoxin type A molecule was determined (Krieglstein *et al.,* 1994).

Botulinum toxin is an extremely potent toxin causing muscle paralysis. In sufficient amounts, the toxin causes death, usually within a few days, from respiratory failure. However, minute amounts of this potent neurotoxin were discovered in 1974 to actually have beneficial effects for persons with some movement disorders. FRI scientist, Dr. Schantz, contributed greatly to this pharmacological research by supplying purified toxin for experiments and clinical trials (Scott, 1981; Schantz and Johnson, 1997).

D. *Clostridium perfringens*

Although the pathogenicity of *C. perfringens* was indicated by observations on diarrheal outbreaks as long ago as 1899, it was not until the late 1950s that this bacterium was confirmed as a pathogen by some volunteer feeding studies. Unlike food poisoning due to *C. botulinum* which results from bacterial growth and toxin production in foods, illness caused by *C. perfringens* results from ingestion of bacterial cells in foods followed by sporulation and enterotoxin production in the intestine. Research by Dr. Dorothy Strong and Dr. Charles Duncan and their associates at FRI contributed significantly to our knowledge of growth *C. perfringens* in foods, to the development of methods for its detection, and to our knowledge of factors affecting enterotoxin synthesis and activity (Duncan and Somers, 1972; Duncan *et al.,* 1972; Strong and Ripp, 1967). The enterotoxin was first purified by affinity chromatography and crystallized at FRI (Scott and Duncan, 1975). Some work on the

mechanism of action of this enterotoxin continued through the early 1980s (DasGupta and Pariza, 1982).

E. Viruses

Hepatitis A and polio viruses were recognized causes of illness by 1946 and some reports indicated that they could be foodborne. However, data on these early outbreaks was incomplete and not widely disseminated. As more evidence of transmission of viruses by milk, cream, and shellfish was publicized, research in this area intensified. Dr. Dean Cliver joined FRI in 1962 and initiated a research program focused on detection of viruses in foods by immunological methods. With the aid of the virology section at FRI, the World Health Organization (WHO) established a Food Virology Program in 1969. During subsequent years research continued on the development of better methods for virus detection and serotyping, survival of viruses in wastewater, and factors causing inactivation of viruses, particularly during food processing (Cliver, 1986).

F. Mycotoxins

Moldy grains, in particular those infected by the ergot fungus, *Claviceps purpurea,* have been known to cause illness in people in Europe since at least the 1200s. Research in the 1900s identified some of the toxic compounds responsible for ergotism. However, there was not much interest in or research on the toxins produced in foods by other fungi until the 1960s. At this time, reports became available which related the plight of thousands of Russians who died during World War II after eating bread made from moldy millet infected with *Fusarium* spp. and of thousands of turkeys and other animals that died in England in 1961 after consuming moldy peanut meal. Isolation of aflatoxin from these peanuts infected with *Aspergillus flavus* focused attention on mycotoxins as causes of foodborne illness in humans (Asao *et al.,* 1963).

Dr. F. S. Chu conducted a vigorous research program on aflatoxins and other mycotoxins at FRI for over 30 years. He and his co-workers developed enzyme-linked immunoassays, radioimmunoassays, and monoclonal antibodies which enabled the detection of aflatoxins and their metabolic precursors as well as other mycotoxins including fumonisins and cyclopiazonic acid (Dorner *et al.,* 2000; Hsu and Chu, 1995; Lee and Chu, 1999; Liu *et al.,* 1997; Yu and Chu, 1998, 1999). These immunological techniques were used to investigate the biosynthesis of mycotoxins as well as chemicals or treatments which might alter their toxic effects.

Occurrence and effects of other mycotoxins were also investigated (Park and Chu, 1996).

G. *Bacillus cereus*

Bacillus cereus produces both an emetic toxin during growth in cooked rice and pasta and a diarrheal enterotoxin during growth in meats, vegetables, puddings, and sauces. The first well-documented *B. cereus* outbreak in the United States, traced to contaminated meat loaf in 1969, stimulated interest in this pathogen. Research at FRI has been directed primarily toward detection, purification, and characterization of the enterotoxins produced by *B. cereus.* Early research demonstrated that the diarrheal toxin was a true enterotoxin (Spira and Goepfert, 1975). In recent years, Dr. Wong characterized hemolysin BL from *B. cereus* and helped define its toxic effects (Beecher and Wong, 2000; Beecher *et al.,* 2000; Schoeni and Wong, 1999; Su and Wong, 1998). Growth of *B. cereus* in foods, particularly cheese, milk, and eggs, has also been investigated.

H. *Campylobacter jejuni*

This bacterium has long been known as a cause of human diarrhea but did not attract much attention as a foodborne pathogen until the 1980s. Studies were initiated at FRI and other institutions to investigate prevalence and growth of *C. jejuni* in dairy products, chicken, and other foods (Doyle and Roman, 1982; Stern *et al.,* 1985). *Campylobacter jejuni* appears to be a common contaminant of poultry and, in recent years, has been estimated to cause more cases of foodborne illness than *Salmonella* spp. (Mead *et al.,* 1999). Research on this organism has been directed at understanding its virulence, its attachment to surfaces, and methods to prevent its establishment in chickens (Ketley, 1997). Although *C. jejuni* usually causes a transitory mild illness, in some cases it persists to cause chronic illness, such as arthritic symptoms and Guillain-Barré syndrome (Smith, 1995).

I. *Escherichia coli*

Escherichia coli has been a recognized cause of infant diarrhea since the 1880s but the association of this bacterium with foodborne illness came many years later. Four types of *E. coli* have been shown to cause diarrhea: enteropathogenic, enterotoxigenic, enteroinvasive, and enterohemorrhagic. Although the hemorrhagic strain, *E. coli* O157:H7, is the most widely publicized in the United States because of its severe consequences in young children, other types of *E. coli* are important

causes of diarrhea in traveling tourists and also in children living in many developing countries. Scientists at FRI have been involved in research on this organism with much of the early work done on enteropathogenic strains. Genetic evidence now indicates that *E. coli* O157:H7 evolved from an enteropathogenic strain by acquisition of a number of virulence genes (Feng *et al.,* 1998). Since this organism caused two large disease outbreaks traced to ground meat in 1982 (Wells *et al.,* 1983), efforts have been concentrated on the study of its toxins and other virulence factors, methods for its detection, attachment of bacteria to food preparation surfaces, and methods for killing or preventing growth of this pathogen (Doyle and Schoeni, 1984; Padhye *et al.,* 1989; Weeratna and Doyle, 1991; Glass *et al.,* 1992; Farrell *et al.,* 1998; Ansay and Kaspar, 1997; Faith *et al.,* 1998). Investigators have also monitored some dairy farms to assess potential routes for dissemination of *E. coli* O157:H7 (Shere *et al.,* 1998).

J. *Listeria monocytogenes*

In contrast to *E. coli, Listeria monocytogenes* seemed to appear suddenly in the United States and Canada as a new foodborne pathogen about twenty years ago (Fleming *et al.,* 1985; Schlech *et al.,* 1983). In actuality, it probably caused many undiagnosed human cases of foodborne illness previously and certainly was known to be an animal pathogen for many decades. By the mid-1980s, research on *L. monocytogenes* was in high gear with particular emphasis on the detection and growth of this pathogen in milk and dairy products (Doyle *et al.,* 1987; Ryser and Marth, 1987). Various inhibitory substances, including bacteriocins, were tested for their ability to limit growth of this pathogen (Buyong *et al.,* 1998). Most recently, the presence of *L. monocytogenes* on ready-to-eat foods, particularly meats, has been the cause of some outbreaks and numerous recalls of products. Therefore, research has expanded to determine reliable methods for limiting or preventing growth of this pathogen in sausage, hot dogs, and other meats (Wang and Johnson, 1997). Although listeriosis is usually a mild disease, pregnant women, the aged, and the immunocompromised may be severely affected as *L. monocytogenes* passes out of the intestine to disrupt other body functions. Mechanisms involved in this virulence are an ongoing, important research area.

K. Seafood and Algal Toxins

Some seafood and algal toxins have been of interest to FRI researchers since the 1970s. Immunochemical studies of saxitoxin, neosaxitoxin, tetrodotoxin, and microcystins have led to the development of several

immunochemical assays for their detection in foods (Chu *et al.,* 1996; Gilroy *et al.,* 2000; Huang *et al.,* 1996).

L. Other Microbes

In addition to these major foodborne pathogens, there are other foodborne bacteria of some concern, such as *Arcobacter* and *Yersinia enterocolitica.* Still other organisms, which are potentially foodborne, may be significant causes of illness including: *Helicobacter pylori,* the bacterium associated with ulcers; *Mycobacterium paratuberculosis,* which may be associated with Crohn's disease; some protozoa; rotaviruses and Norwalk virus; and the agent responsible for bovine spongiform encephalopathy (BSE). In the past 10 years some new foodborne pathogens have been added to the list, but more importantly, the conceptual framework and the scientific approach for investigation of foodborne disease has undergone transition. Increasingly there is emphasis on determining pathogen adaptations to environmental stress and the cross-species, cross-genus transfer of genetic material.

III. Future Prospects and Perspectives

What about the future? Will new pathogens emerge? What foodborne disease issues are likely to be important in the next decade? Of course it is impossible to tell for sure, but analysis of some trends in recent foodborne outbreaks can give us clues.

A. Foodborne Pathogen Dispersal

Widespread dispersal of foodborne pathogens has become more obvious in recent years as transportation has become faster and more efficient and increasing amounts and varieties of foods are imported and exported. In the past, an outbreak of foodborne disease was usually confined to one city or a small geographical area. Now, we see the same strain of *Listeria monocytogenes* causing illness in ten states in the United States—from California to Connecticut (Centers for Disease Control, 2000).

"Globalization" of our food supply has ensured that foodborne pathogens, like other infectious agents, are no longer restricted by geography. Therefore, the presence or absence of microbial contaminants on our foods depends on the hygienic practices of the producing countries and of the storage and transporting companies as well as those of the local food stores, restaurants, and consumers. In 1996–1997, several outbreaks of *Cyclospora cayetanensis* affecting more than 1000 people

occurred in the United States and Canada. Previously, *C. cayetanensis* had only been rarely isolated in industrialized countries, although it was known to be more common in underdeveloped countries. Epidemiological investigations tracing the source of *C. cayetanensis* indicated that it was present on fresh fruit and that the most probable food vector was raspberries grown in Guatemala. It appeared that the lack of sanitary facilities for workers and the use of contaminated water for washing contributed to the presence of microbes on the berries (Herwaldt, 2000).

In the early 1990s epidemic cholera reached South America from Asia. It was not long before cholera cases appeared in the United States among airline travelers from South America in 1991 and in persons consuming food imported from South America in 1991 and 1994. In the past decade there have also been outbreaks of shigellosis in the United States traced to imported parsley and of hepatitis A traced to imported strawberries (Centers for Disease Control, 1997b, 1999a).

Other recent examples of long-range transportation of food pathogens occurred with the contamination of shellfish in 1995–1998. Bacteria which cause cholera, *Vibrio cholerae,* were detected in oysters in Mobile Bay, Alabama. These contaminants were traced to ballast water dumped from ships which had been to Peru, then the site of a major outbreak of cholera. In 1998, oyster beds in Galveston Bay, Texas, were closed to harvesting during most of the summer because 416 persons in 13 states fell ill after eating raw oysters traced to this location. The culprit in this case was *Vibrio parahaemolyticus,* a common pathogen in Asia causing numerous outbreaks in Taiwan, Japan, and some southeast Asian countries. The particular strain of *V. parahaemolyticus* involved in this outbreak, O3:K6, had not previously been detected in coastal waters of the United States, although it had been isolated from cases of diarrhea in Asia since 1994. It appears that this virulent strain may also have been introduced to United States coastal waters by ballast water discharged from ships which had traveled to Asia (Centers for Disease Control, 1999b).

At the beginning of the new millenium, the spread of bovine spongiform encephalopathy (BSE) is a major concern. The disease was initially diagnosed in cattle in Great Britain in 1987 and at first appeared to be confined to that country. However, many other European countries have now detected BSE in native-born cattle. In 1996, ten human cases of a variant of Creutzfeldt-Jacob disease (vCJD), apparently linked to BSE, were reported in Great Britain. So far the Department of Health in the United Kingdom has identified a total of 102 cases of vCJD as definite and probable as of the end of June 2001. Many countries around the world have banned imports of beef from European countries which have reported cases of BSE (Brown *et al.,* 2001).

B. Changing Food Preferences

Food preferences have changed during the past few decades, with consumers seeking more convenient foods for busy lifestyles, minimally processed foods, and fresh produce all year round. In fact, demands for fresh produce during winter and spring is one of the driving forces for importation of these foods from tropical areas and the Southern Hemisphere. However, it is not only imported foods which have harbored the microbes causing recent outbreaks. Fresh, unpasteurized juices and cider produced in the United States, contaminated with *E. coli* O157:H7 and *Salmonella muenchen,* caused notable outbreaks in 1993 and 1996 (Besser *et al.,* 1993; Centers for Disease Control, 1996b, 1996c). *Escherichia coli* O157:H7 on sprouts and items at salad bars in restaurants have also caused foodborne outbreaks during the past 5 years (Centers for Disease Control, 1997a). Some of these foods apparently had been contaminated in the field while others were contaminated during food preparation.

Ready-to-eat meats, including hot dogs and deli turkey meat, consumed without reheating, have also been carriers of *Listeria monocytogenes,* which is capable of growth at refrigeration temperatures. Outbreak-related cases were identified in many states (Centers for Disease Control, 1998, 2000). Although the microbial pathogens in these foods were often well known, methods for producing, storing, packaging, or preparing these convenient, minimally processed foods were not adequate for preventing growth of foodborne pathogens.

C. Virulence

Increased virulence has been another hallmark of many recent outbreaks of foodborne disease. Are people becoming more susceptible to these microbes or have the microbes become more virulent? It is likely that both have occurred. Time and again when we examine data from foodborne outbreaks to determine which victims experienced the gravest illness, we find that, for the most part, they were old, very young, or had some underlying disease. In other words, their immune systems were weakened and unable to cope effectively with the infection. As our population ages and more people survive heart attacks, cancer, and organ transplants, there are more immunocompromised people and a greater likelihood that *Listeria monocytogenes, Vibrio parahaemolyticus, E. coli* O157:H7, and other pathogens will cause life-threatening foodborne disease.

Some microbes have also become more virulent by acquiring genes for toxin production from other species of bacteria. Until 15 years ago,

foodborne botulism was thought to be caused only by *Clostridium botulinum.* Now outbreaks of botulism in such diverse areas as China, Italy, and India have been traced to *C. butyricum* and *C. baratii* isolates producing botulinum toxins (Chaudhry *et al.,* 1998; Fenicia *et al.,* 1999; Meng *et al.,* 1997; Schechter and Arnon, 1999; Suen *et al.,* 1988). Laboratory investigations revealed that the toxin gene from at least one strain of *C. botulinum* was most likely transferred to *C. butyricum* by a lysogenic bacteriophage (Wang *et al.,* 2000; Zhou *et al.,* 1993).

Escherichia coli O157:H7, the cause of numerous food- and waterborne outbreaks in the last decade, appears from genetic analyses to have evolved from an enteropathogenic strain of *E. coli* which acquired genes for production of Shiga toxins by viral transduction (Feng *et al.,* 1998; Reid *et al.,* 2000). The recently published genetic sequence of the *E. coli* O157:H7 chromosome demonstrates that this organism differs from a common, nonpathogenic strain of *E. coli* by having more than 1000 additional genes (Perna *et al.,* 2001). Clusters of genes related to virulence were detected at several sites on the chromosome, suggesting that these clusters may have been acquired as units from other bacteria—perhaps with the aid of viruses. Transfer of virulence genes from *E. coli* to *Salmonella* in membrane vesicles has also been observed (Yaron *et al.,* 2000).

D. Microbial Adaptations

Physiological adaptation to cope with stress, coupled with naturally occurring horizontal gene transfer, is evident in recent studies of foodborne pathogens. The microbe's "goal" is not to kill its human hosts but rather to survive and reproduce. An examination of the ecology of foodborne bacteria reveals that there are several abiotic and biotic factors that affect survival. Traditionally, we have made use of many of these limiting factors by ensuring that our foods are too cold, hot, salty, spicy, or acidic to permit pathogen growth. Animals and sometimes fruits intended for human consumption are often treated with antibiotics which drastically reduce bacterial numbers. Nevertheless, large numbers of bacteria present in the environment, coupled with their genetic diversity and rapid growth, enable some populations to survive and adapt to these environmental insults. Although 99.99% of a microbial population may be killed by some restrictive environmental condition, the survivors carrying their own resistance genes or genes acquired from other bacteria will persist and multiply in the niche created by the presence of an antibiotic or acid. These problems have become particularly evident in studies of biofilms on food processing surfaces (Farrell *et al.,* 1998; Wong, 1998) and the development of acid tolerance by *E. coli*

O157:H7 (Cheng and Kaspar, 1998; Choi *et al.,* 2000). Resistant microorganisms have been selected for in many inhospitable environments and are now more difficult to control.

Some bacterial pathogens have become resistant to several antibiotics—witness, for example, the dramatic rise of *Salmonella typhimurium* DT104, a strain resistant to five or more antibiotics (ampicillin, chloramphenicol, streptomycin, sulfonamides, tetracycline, and sometimes trimethoprim, spectinomycin and/or ciprofloxacin) (Threlfall, 2000). Another foodborne bacterium, *Campylobacter jejuni* from chicken, has apparently become increasingly resistant to fluoroquinolines following the introduction of these drugs for use in poultry (Smith *et al.,* 1999). Researchers in Denmark have monitored changes in bacterial antibiotic resistance in the human population and have found correlations with antibiotic usage in food animals (Aarestrup, 2000).

Other microbes that are normal residents in the environment can also affect the activity of foodborne pathogens. *Salmonella enteritidis* was once a relatively rare bacterium usually associated with rodents. Starting in the 1960s, chickens were identified as hosts for *S. enteritidis,* and human illness traced to infected poultry and eggs started to increase. By 1988, 15,427 human cases of *S. enteritidis* were reported in England and Wales, and in 1997 the epidemic appeared to peak with 7924 cases of *S. enteritidis* reported by CDC in the United States and 23,008 were reported by the Public Health Laboratory Service in England.

Why and how did this former recluse rise to such prominence? Some recent research traced the rise of *S. enteritidis* and demonstrated once again that tinkering with Mother Nature can have unexpected consequences. Since mice and rats carrying *S. enteritidis* forage for food around henhouses, there is a ready source of infection for chickens, but this bacterium was rarely detected in chickens before the 1960s. However, prior to the 1970s, chicken flocks were often infected with *S. pullorum* and *S. gallinarum,* two species causing serious illness in poultry and economic losses to the poultry industry. Both England and the United States undertook aggressive campaigns to rid chicken flocks of these two pathogens and largely succeeded by the mid-1970s. With the demise of *S. pullorum* and *S. gallinarum,* an ecological niche was opened up, and *S. enteritidis* took advantage of it. Because *S. enteritidis* did not cause illness in poultry, it was not noticed at first. However, it soon became an important contaminant of eggs. This strain can even be detected inside some hens' eggs as it can be passed from the hen into eggs prior to laying (Ebel and Schlosser, 2000).

Recent data on the incidence of *S. enteritidis* infections in humans reveals a distinct downward turn since 1997. As yet, there is no definite explanation for this fall in numbers of *S. enteritidis.* Most likely, both

greater consumer awareness of the importance of cooking eggs properly and greater efforts on the part of the poultry industry to produce cleaner birds and eggs have contributed to the decrease in cases of *S. enteritidis.* While this trend may continue, we must be vigilant for the next challenge issued by foodborne pathogens.

Competitive effects of harmless bacteria can be used to advantage by poultry producers and food processors. Feeding of newly hatched chicks with doses of normal chicken intestinal bacteria has effectively prevented colonization by pathogenic salmonellae in some experiments (Bailey *et al.,* 2000; Mead, 2000). Similar results have been obtained with normal cecal bacteria restricting the growth of *C. jejuni* in chicks (Schoeni and Doyle, 1992). Some of these competitive exclusion cultures have been commercialized and may reduce the incidence of salmonellae and campylobacter on chicken. Competitive lactic acid bacteria and some of their bacteriocins have also been used to prevent or reduce growth of *L. monocytogenes.* Some recent reviews summarize results of experiments using bacteriocins to control *L. monocytogenes* in foods and discussed modes of action of these compounds, factors affecting their effectiveness, and development of resistance in *L. monocytogenes* (Aymerich *et al.,* 1998; Hugas, 1998; Muriana, 1996).

IV. Conclusions

Perhaps the overriding lesson we should learn from our recent experiences with foodborne disease is that we need to adopt a more holistic approach to the study of food safety. Identifying the culprit in a foodborne disease outbreak is only the first step. We also need to understand how and why this organism has became a problem. Has it become more resistant to chemical preservatives or preservation conditions that previously limited its growth? Has adaptation to acidic or salty conditions made the bacterium more resistant to the lethal effects of heat? What is the genetic basis of resistance and where did it arise? Did the pathogen pick up virulence or antibiotic resistance genes from some other species? Were there changes in food preparation or processing methods which allowed growth of the pathogens? Did the microbes originate on the farm, e.g., in contaminated cattle or vegetables?

As we endeavor to maintain human health and produce and package nutritious foods, we have devised new and more effective methods for the prevention of foodborne illness. However, we are constantly challenged by examples of nature "fighting back." In order to effectively prevent foodborne disease now and also predict emerging trouble spots in the future, we should be aware of as many aspects of the ecology of these organisms as possible. Only then can we make informed

decisions for appropriate modifications in food production, preparation, and preservation techniques.

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Alexander Fleming and the Discovery of Penicillin

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I. Introduction

As microbiologists look back on the last century and reflect on the implications of our science, the discovery of penicillin remains one of the most important milestones. Penicillin permanently changed infectious disease research and therapeutic medicine, it transformed patient expectations and the structures of drug companies, it contributed new insights in microbiology and molecular biology, and moreover, it captured the public imagination as did few other scientific breakthroughs. Sometimes called the "single greatest victory ever achieved over disease," penicillin is inextricably linked with the name of Alexander Fleming, the scientist who discovered an antibacterial activity in a *Penicillium* growth medium. To avoid repetition of the phrase "mold broth filtrate," he coined the term "penicillin," a substance that eventually became known as a "miracle drug." Although Fleming's story has been told many times—and with enough elaboration that it is sometimes called the "Fleming myth"—the story retains a compelling fascination. And as with all good stories, each teller, each listener, and each era finds new meanings and interpretations.

Most of the narrative material in this essay is abstracted from two major biographies: *The Life of Sir Alexander Fleming, Discoverer of*

ADVANCES IN APPLIED MICROBIOLOGY, VOLUME 49

0065-2164/01 $35.00

Penicillin (Maurois, 1959) commissioned by Fleming's widow after his death, and *Alexander Fleming, the Man and the Myth* (MacFarlane, 1984) written by Howard Florey's major biographer.

II. Fleming's Early Years

Alexander Fleming was born on August 6, 1881, at Lochfield Farm in Ayrshire, Scotland. His father was Hugh Fleming, who had been a widower of sixty with four children when he married Grace Morton in 1876. Alexander (Alec) was his father's seventh child, and his mother's third. Eventually there were eight Fleming children. They remained a close knit family throughout life.

Alec Fleming was just seven when his father died. His oldest half brother, Hugh, took over the running of the family farm, providing for his younger siblings, with the assumption that all the younger brothers would eventually have to find professions outside of farming.

The family lived amid the harsh beauty of the Scottish countryside, remote from city life, without modern amenities. Fleming's first schooling was in a tiny moorland school and then, at age ten, he transferred to a larger facility in Dorval, a small town four miles from his home. He had to walk to and from school every day, no matter what the weather, and later when he was famous journalists liked to tell of the way he went barefoot in summer, and carried hot potatoes in winter to keep his hands warm. In 1893, Fleming started at the Kilmarnock Academy where he was exposed to a remarkably wide curriculum. Throughout his early school years he performed well with little apparent effort.

When just past thirteen, Alec Fleming went to London where his half brother Thomas was an ophthalmologist, and his older brother John worked for an optical firm in the lens business. His younger brother, Robert, eventually joined them too. The housekeeping was done by various sisters and later by their mother. In London, Alec Fleming attended classes at the Regent Street Polytechnic for two years, and then spent four boring years as a clerk in a shipping company. He did not like the job, and was more than ready to leave when he had a lucky break: In 1901, an uncle died and left him a small legacy. His brother Thomas encouraged him to study medicine. Alec took the advice, although at the time he lacked the educational qualifications. After a short period of studying, he sat for the qualifying exams and not only passed, but tied for first place and secured a scholarship. Of the 12 medical schools in London, he selected St. Mary's Hospital School. Later, he said his decision was based on the fact that St. Mary's had a swimming team, and that the only reason St. Mary's had accepted him was because it was one of London's least distinguished medical schools. Distinguished or

not, St. Mary's became one of the best bacteriological research facilities in England. Sir Almroth E. Wright, a pioneer of the vaccine treatment of disease and an early convert to experimental medicine, had accepted the Chair of Pathology in 1902. Wright devoted much of his considerable energy to the conquest of infectious disease, in particular through the therapeutic use of vaccines. His theory involved phagocytes and was premised on the notion that it was not enough for microbes and phagocytes to be in contact—the microbes had to be "prepared" before the phagocytes could inactivate them. The property in the blood stream that facilitated phagocytosis was dubbed "opsonin." In order to develop specific immunizations against bacterial infections, "opsonic power" had to be measured, a laborious and time-dependent process that required a dedicated staff.

The day after Alec Fleming qualified in medicine, on August 6th, 1906, he began work in St. Mary's Hospital under Wright's directorship. His first solo paper in 1908 concerned the Opsonic Index. The following year, he was awarded a Gold Medal for his success in the Final Fellowship Examination of the Royal College of Surgeons, the last of a series of distinguished academic prizes he had won during his medical school years. An editorial in the *St. Mary's Hospital Gazette* at this time describes him as "one of Sir Almroth Wright's most enthusiastic followers."

Not long after, Paul Ehrlich injected the 606th compound in his famous series in search of an antisyphilitic drug. Modern chemotherapy was born with Compound 606 (later called Salvarsan) and in England it was used first at St. Mary's. Fleming became adept at injecting Salvarsan directly into veins, and was probably the first English physician to use the new chemotherapeutic agent. Developing a reputation as one of the best venerologists in London, he treated many patients, inventing a simple apparatus with two glass jars, a syringe, two rubber tubes, and two taps with double nozzles. This apparatus made it possible to treat four people with Salvarsan at the same time. A cartoon from that era, "Private 606" (Fig. 1), shows a slight, kilt-clad, broken-nosed, high-domed man with a cigarette dangling from his lips. "Little Flem" as he came to be called, was to remain a popular albeit extremely laconic figure throughout his life.

When World War I broke out, Fleming joined the Royal Army Medical Corps, serving under Wright in a wound-research laboratory at Boulogne in France. High explosives were being used extensively for the first time in a military setting. The human waste was appalling. The fractures and mutilations were accompanied by almost universal septic infection. Fleming, working with Wright and Leonard Colebrook, studied the source of wound infections and the best way to

FIG. 1. "Private 606" (from St. Mary's Archives).

treat them. At the time, standard medical practice was based on the principles of Lister, and involved irrigating wounds with antiseptics like carbolic acid and iodine. The wound research unit showed that clothing was a major source of infection. They cultured and identified the most common bacteria causing wound infections. Most controversially, their research demonstrated that antiseptics were better at killing leukocytes than they were at killing bacteria. Fleming used a glass-tube device to mimic the rough surfaces of wounds, and established that antiseptics did not reach all of the bacteria. Ultimately, the Wright group recommended that wounds should be washed with simple hyptertonic saline solutions. Their finding that the Listerian antiseptics were doing

more harm than good was not well received by the medical establishment.

Just before Christmas in 1915, Alec married Sarah (Sareen) Marion McElroy, a vivacious and outgoing woman who ran a nursing home with her twin sister. Alec and Sareen were to have a long and happy marriage, despite—or perhaps because of—their different personalities. Later, Alec's brother John married Sareen's twin, strengthening already strong family ties.

In 1919, demobilized from the Army with the rank of captain, Fleming returned to St. Mary's to continue working on antibacterial mechanisms. He gave up most of his private practice and concentrated on running the Inoculation Department and doing research.

III. Preludes and Penicillin

In 1921 Fleming was suffering from a cold and discovered that something in his nasal mucus caused a clear zone to form around a certain golden-yellow bacterial strain. After demonstrating that the phenomenon was not due to bacteriophages (then a hot topic of research), he named the new bacteriolytic agent "lysozyme" and the susceptible bacterium isolated from his nose *Micrococcus lysodeikticus*. Lysozyme could turn a turbid suspension of sensitive bacteria into a solution as "clear as gin." Working in collaboration with V. D. Allison, Fleming found that lysozyme was a normal component of various animal fluids (serum, tears, saliva, milk) as well as egg whites, certain plant tissues, and a variety of other sources.

Although lacking in obvious therapeutic potential, lysozyme was an important discovery and Wright nominated Fleming—unsuccessfully—to the Royal Society. In fact, the lysozyme research received little recognition. Fleming's poor speaking ability no doubt contributed to the general neglect. Curiously, the only people to follow up were Howard Florey and Ernst Chain, who purified the enzyme in 1937. Notwithstanding, Fleming's later observation of bacterial inhibition by a mold was so similar to his observations of bacterial inhibition by nasal mucus that the discovery of penicillin has become the canonical illustration of Pasteur's celebrated aphorism that "In the field of experimentation, chance favors only the prepared mind" (as quoted in Dubos, 1976).

Almost all versions of the penicillin saga include Fleming's lysozyme work, but another important chapter is often omitted. Fleming had become an expert in the treatment of boils, a nonlethal infection caused by *Staphylococcus aureus*. During the 1920s, the Medical Research Council asked him to prepare the section on *Staphylococcus* for their

nine-volume treatise, *A System of Bacteriology*. Fleming was doing research in preparation for writing his contribution when the famous laboratory accident occurred.

The story has been told and retold in slightly different variations. In September of 1928, Fleming returned from a vacation and looked through some old Petri plates to discover that a contaminating blue-green mold had created a clear, halo-like zone around a colony of *Staphylococcus*. For some considerable distance around the mold, the staphylococci were undergoing lysis with an uncanny similarity to lysozyme. Unlike lysozyme, however, the fungus was lysing a *pathogenic* bacterium.

Fleming recorded the event in his lab book. He showed the plate to several co-workers, photographed it, and made it permanent by exposing it to formalin vapor. The original plate is now in the British Museum. A mycologist at St. Mary's (mis)identified the mold as *Penicillium rubrum* and Fleming named the mold broth filtrate "penicillin." In further studies, he established cultural conditions for producing the antibacterial substance, described the selective inhibitory effect of penicillin on different bacterial species, demonstrated its nontoxicity to animals, showed that even when the mold juice was diluted 800 times it prevented the growth of staphylococcus, and made a number of unsuccessful attempts to purify the crude extracts. The paper describing this work was published in the *British Journal of Experimental Pathology* in June of 1929 and included mention of the possible clinical applications of penicillin (Fleming, 1929). A chronology of important dates in the development of penicillin is presented in Table I.

It is now known than penicillin controls bacteria by inhibiting their cell division. Hence, the phenomenon observed by Fleming—lysis—was extremely unusual. Many years later, both Ronald Hare (Hare, 1982) and Milton Wainwright attempted to duplicate Fleming's observation. Wainwright's book, *Miracle Cure, The Story of Penicillin and the Golden Age of Antibiotics* (1990) describes both sets of experiments in considerable detail. In a nutshell, although it is relatively easy to demonstrate bacterial inhibition, the bacterial lysis that caught Fleming's attention on the famous Petri Plate could only have been due to an extremely unusual set of events. After years of research on lysozyme, Fleming was the perfect eyewitness for this unlikely microbiological contingency. Moreover, Fleming, who had strong belief in the curative powers of natural substances, was a receptive observer. Lysozyme was an enzyme that dissolved microorganisms. Antiseptics like carbolic acids were applied externally and killed microorganisms directly; chemotherapeutic agents like Salvarsan—and the sulfa drugs—were synthetic chemicals that were injected and killed microorganisms systemically. Penicillin

TABLE I

CHRONOLOGY OF EVENTS IN EARLY PENICILLIN RESEARCH[a]

Sept. 3 or 4, 1928: **Fleming discovers a halo of lysed bacteria surrounding a mold** colony.

Jan. 9, 1929: Fleming unsuccessfully treats Stuart Craddock's chronically infected nasal antrum.

May 10, 1929: **Fleming submits paper to *British J Experimental Pathology* (publication June, 1929)**(Fleming, 1929)

Aug.–Dec. 1930: C. G. Paine treats eye infections in three newborns. (Does not publish his findings but clinical records were later recovered. [Wainwright and Swan, 1986]).

1932: Fleming successfully uses penicillin to treat an eye infection in K. B. Rogers, a member of the laboratory. Paine discusses his penicillin work with Howard Florey. Florey shows "not the slightest interest at that time" (Wainwright and Swan, 1986). Raistrick's group reports partial purification of penicillin at Biochemical Society Meeting (Clutterbuck *et al.,* 1932).

1934–1935: Roger D. Reid (Pennsylvania State) surveys a group of molds for penicillin production and confirms both bacteriostatic effect and chemical instability (Reid *et al.,* 1934, 1935).

1935: Florey moves to Oxford to become the Sir William Dunn Professor of Pathology; Ernst Chain joins Florey's group.

Jan 27, 1939: Florey applies to Medical Research Council for "continuation of work on lytic substances."

May 25–26, 1940: **First mouse experiments at Oxford.**

Aug. 24, 1940: **First publication on penicillin as a chemotherapeutic agent** (Chain *et al.,* 1940).

Oct. 15, 1940: Oxford results confirmed by Henry Dawson, working at Columbia Presbyterian Hospital in New York, who is first to inject human patients with penicillin (Dawson *et al.,* 1941).

January–June 1941: Heatley and associates enhance penicillin production; Oxford group treats six patients with staphylococcal and streptococcal infections (Abraham *et al.,* 1941).

July 1941: Florey and Heatley arrive in New Haven, CT, on July 2nd, and in Peoria, IL, on July 14th Florey quickly returns to Britain; Heatley stays until November "passing on the secrets of penicillin production" to the Peoria group.

1942: Dr. M. Ethel Florey treats over 170 patients with penicillin (Florey and Florey, 1943).

March 14, 1942: Anne Miller, wife of a Yale administrator, is dying of a puerperal infection of hemolytic streptococcus; she is treated with penicillin obtained from Heatley and Dr. Max Tischler at Merck. First successful cure in USA (Urtz, 1985).

TABLE I—*Continued*

Aug. 13–20, 1942: Fleming injects Oxford purified penicillin intrathecally into the spine of Harry Lambert (an associate of his brother Robert), who is dying of meningitis. Lambert recovers.
August 27, 1942: Article in *The Times* (London) focuses on penicillin and "miracle cures."
August 31, 1942: Almroth Wright writes letter to *The Times* naming Fleming as the discoverer of penicillin.
Sept. 1, 1942: Sir Robert Robinson writes letter to *The Times* drawing attention to Florey and the work in Oxford.

[a] Most significant events in boldface.

For citations not referenced specifically in the table, see Mcfarlane (1979, 1984), Hobby (1985); and Wainwright (1990).

was something new: a *natural* product of unknown structure with strong antibacterial activity. Some critics with 20–20 hindsight believe that Fleming, who uniquely had triple experience with lysozyme, topical antiseptics, *and* Salvarasan, should have done the "obvious experiment." They wonder why penicillin was not immediately injected into infected animals and tested for its systemic activity. Such retrospective judgment overlooks the fact that at the time of its discovery, Fleming believed that he had discovered a natural antiseptic and that penicillin killed bacteria by lysing them, i.e., by breaking them open. The prepared mind is a double-edged sword. Lysozyme research informed Fleming's observational acuity, but also guided his inferences about penicillin's therapeutic potential. Fleming apparently categorized penicillin as a topical antiseptic. Apart from a few experiments in which "mold broth" was injected into healthy rabbits, all the earliest applications involved external applications. For example, Fleming irrigated the nasal passages of one of his associates, Stuart Craddock, in January 1929. There was no apparent effect and Dr. Craddock's nasal antrum remained infected. Fleming used penicillin again later to clear up an eye infection in a member of his rifle team, Dr. K. G. Rogers. This treatment was successful but Fleming did not publish on this accomplishment (Hare, 1982). There is also recent evidence that Dr. C. G. Paine, a St. Mary's graduate, successfully used penicillin in 1930 to treat eye infections in human patients in Sheffield. Dr. Paine neither published nor pursued this research, but clinical records have survived. Moreover, he mentioned the work to Howard Florey (Wainwright and Swan, 1986)

Another set of hindsight judgments comes from medical historians and popular writers who have uncovered evidence that molds—or

moldy foodstuffs—have been used for therapeutic purposes for centuries. The Ancient Chinese used them to treat boils; American Indians used them to dress their war wounds. Irish grandmothers, Finnish midwives, Mayan priests, African healers, Asian wise men, and witch-doctors of all kinds have all been awarded retroactive priority in penicillin discovery. Undoubtedly fun to read, these anecdotal reports have become a mainstay in certain kinds of fictional anthropological writing. However, since fungi produce many antibacterial substances, it is impossible to know if any of the "Old Mold" Legends actually involved a penicillin-producing *Penicillium*. Virtually all contemporary writers concede that Alexander Fleming deserves full credit for the scientific discovery of penicillin (Wainwright, 1988; Aldridge, 2000).

IV. The Lull before the Storm

In 1932, Harold Raistrick, then at the London School of Hygiene and Tropical Medicine, continued research on Fleming's *Penicillium*. At that time, Raistrick was the leading chemical expert on fungal metabolites. Questioning the taxonomic identification of the mold, he sent a subculture to Charles Thom in the U.S. Department of Agriculture, who ascertained that Fleming's fungus was actually *Penicillium notatum*. Raistrick grew the mold on a defined medium, confirmed the insidious instability associated with purifying penicillin from the mold juice, and then focused his attentions on the analysis of a more tractable accompanying yellow pigment (chrysogenin) (Clutterbuck *et al.,* 1932; Bentley and Thomas, 1990). At the 1935 International Congress of Physiologists in Leningrad, Raistrick was quoted as saying that he "thought the production of penicillin for therapeutic purposes was almost impossible" (quoted in Wainwright, 1990, p. 31). Fleming lacked chemical expertise, and did not pursue the purification of penicillin after the leading expert in the field had tried and failed. Instead, his laboratory provided cultures to those who requested them, and continued to produce penicillin on a small scale and to use it for the differential cultivation of certain bacterial species as part of the St. Mary's vaccine development work. With the exception of Roger Reid, a graduate student at Pennsylvania State College (now University) who surveyed a number of molds for "Fleming's antibacterial substance", (Reid, 1934, 1935), and Siegbert Bornstein, working at Beth Israel Hospital in New York, who tested the action of penicillin against a number of Enterococci (Bornstein, 1940), little interest was shown in penicillin during the ten years after its discovery.

V. The Oxford University Group

Fleming played almost no part in the development of penicillin as a drug. The basic work was done at Oxford University where penicillin was first purified and produced in sufficient quantity for early clinical trials. The credit for initiating the reinvestigation of penicillin goes to Ernst Chain, while it was Howard Florey who had the vision, force of character, and administrative expertise not only to hold together the team that demonstrated penicillin's sweeping power to cure infectious diseases but also to interest the American government in this potential. See Florey and Abraham (1951) and MacFarlane (1979), Clark (1985), and Kiester (1990) for more detailed descriptions of the events summarized below.

Florey was an Australian-born Rhodes scholar, recently moved to Oxford from Sheffield. He had a strong interest in bacterial antagonism, and while working with Ernst Chain, a German-born émigré, purified lysozyme in 1937. With the intent of finding other promising antibacterial substances, Chain conducted an exhaustive literature review, turning up more than 200 leads. From this group, Chain and Florey decided to focus on penicillin. It is difficult to know how much their earlier experience with Fleming's lysozyme contributed to their decision. Chain later said that he started out thinking that penicillin was an unstable enzyme, "a sort of mould lysozyme." Both Chain and Florey made it clear that their original aspirations were purely biochemical. Florey was later quoted as saying, "I don't think that the idea of helping suffering humanity ever entered our minds."

It was agreed that Florey would do the biological experiments while Chain would study the chemical and biochemical properties of the mold metabolite. The first hurdle was to obtain sufficient penicillin to conduct experiments. A third key player of the Oxford group was Norman Heatley, who was put in charge of the microbiological aspects of the project (Moberg, 1991). Self-effacing and retiring, Heatley's personality was diametrically different from the extroverted and opinionated Chain. Heatley agreed to participate only if he could report directly to Florey. Nevertheless, despite their early and continuing conflicts, these three men worked together to perform the groundbreaking experiments. Heatley grew the mold, Chain extracted penicillin from the broth, and Florey supervised the animal trials and kept an ever-enlarging and frequently bickering group together as a functioning unit.

The pivotal experiment was performed on Saturday, May 25, 1939, as British troops were massing for the evacuation at Dunkirk. Eight mice were injected with a virulent strain of streptococcus. Four of the mice were left as untreated controls; the other four were injected with

penicillin. Heatley stayed with the mice through the night. By 3:30 the following morning, the control mice all had died; the penicillin-treated mice all were alive and Heatley bicycled home. On Sunday, when Chain, Florey, and Heatley gathered to discuss their results, there is no record of what—if anything — Heatley said. Florey commented, "It looks quite promising," while Chain proclaimed, "A miracle." Over the next months, the experiments were repeated and expanded. The paper reporting on the animal trials was published on August 24, 1940 in *The Lancet* (Chain *et al.,* 1940). Almost immediately there was a response in New York. Henry Dawson, a clinician with a strong interest in bacterial endocarditis, read the August 24, 1940 paper in *Lancet,* obtained a culture of *P. notatum* from Roger Reid, and aided by Gladys Hobby and Karl Meyer, within two months had purified enough crude penicillin to treat two patients. Dosages were too low, and although there were no toxic effects, neither were any cures effected. Dawson died in 1945 unrecognized as the first man to have used penicillin systemically in a human patient (Hobby, 1985; Bentley, 1993).

Meanwhile, back in England, the Oxford group was moving ahead with greater mastery. After trying and failing to get industrial support for his penicillin work, Florey decided to turn his own department at Oxford into a penicillin-manufacturing plant. Using surface culture fermentation in special containers modeled on hospital bedpans, and with the help of six "penicillin girls" paid by the Medical Research Council, a classroom was turned into a cultivation facility. Other rooms and laboratories in the William Dunn School of Pathology became extraction and purification rooms. Heatley ingeniously devised equipment for both cultivation and extraction, as well as an assay method (Moberg, 1991). Chain, working with a new chemist member of the group, Edward Abraham, improved the purification methods and began accumulating enough penicillin for human clinical trials. After a simple toxicity test in a woman dying from cancer, Florey's first patient, Albert Alexander, who had severe staphylococcal infection, was treated on February 12, 1941. There was a period of dramatic improvement, and a second patient was started on February 22nd. The second patient recovered. Unfortunately, when Mr. Alexander relapsed, the penicillin had all been used up, and he died. Over the next few months, more penicillin was produced—through enormous effort—and four more cases of previously incurable disease were treated (Kiester, 1990). Three patients recovered and one died from an unrelated cause. The work was published in August 1941 in *The Lancet* (Abraham *et al.*, 1941).

Florey clearly understood he had a compound of unprecedented medical value and he knew he had to find a way of making more penicillin. A month's work went into isolating enough to treat a single patient.

Increasing the pitifully small yields of the mold metabolite would require resources beyond the capability of the Oxford laboratory, but there was little interest from British industry. Moreover, Britain was at war. Between the devastation of the London blitz and the threat of even more widespread Luftwaffe bombings, the necessary scale-up research would be difficult in England. Florey decided to bring penicillin to the USA, obtaining funds from the Rockefeller Foundation for his trip and choosing Norman Heatley to accompany him. Chain did not learn of these plans until the day of departure. Although Florey's actions may have been motivated by a need to maintain strict wartime security, his behavior toward Chain was insensitive at best. Chain never forgave Florey (Clark, 1985).

Flying first to Portugal and then to New York, Florey and Heatley went directly to New Haven, where the Florey children were spending the war with John Fulton, another former Rhodes scholar and an old friend of the Florey family. Fulton started a chain of contact that led through Charles Thom, the Principal Mycologist of the U.S. Department of Agriculture (and at the time President of Society of American Bacteriologists, now the American Society of Microbiology), and then eventually to Orville May, director of the then new U.S. Department of Agriculture facility in Peoria, Illinois. Florey and Heatley brought the mold and their expertise to Peoria. Florey returned almost immediately to England; Heatley stayed in Peoria sharing everything he knew about culturing the penicillin-producing strain.

When Florey returned to England, he hoped that the USA would soon be providing him with enough drug to continue his clinical studies. The Americans disappointed Florey—the first shipment from Merck did not arrive until April 1942—so most of the penicillin tested by the Oxford Group had to be produced by them, with a modicum of help from some British companies. It is a testament to Florey's organizational genius that his group manufactured penicillin in spite of the shortages and dangers imposed by the war. They developed strategies for maximum impact of a small amount of drug and also recycled penicillin from the urine of treated patients. Dr. M. Ethel Florey, Howard Florey's first wife, took a major role in managing the clinical trials. She was seen regularly in Oxford, riding her bicycle to collect urine from penicillin-treated patients for recycling, a daily ritual dubbed the "P-Patrol." During late 1941 and throughout 1942, Ethel and Howard Florey administered penicillin to over 170 patients. When their results were published in *Lancet* in March of 1943, they demonstrated beyond a doubt that penicillin was an unprecedented advance over any antibacterial substance ever before discovered (Florey and Florey, 1943).

While the Floreys pursued their medical research, Chain and Abraham developed methods for the isolation and purification of penicillin; and with many other groups they attempted the elucidation of its structure (Thomas, 1990). Chain refused to think that any chemical problem was too difficult; his energy and originality also generated a "contagious enthusiasm," a psychological contribution that is impossible to measure. Eventually, Chain sought permission to patent some of the purification methods he and his co-workers had devised. Both Sir Edward Mellanby, Secretary of the Medical Research Council, and Sir Henry Dale, President of the Royal Society, were dismissive and replied that the discovery was for the benefit of mankind—that it was immoral to seek patent protection. Several American scientists, operating under a more capitalistic ethic, did not have similar scruples. After World War II was over, the British were dismayed that they might have to pay royalties for using cultivation methods on what was rightly believed to be an English discovery. Chain's regular and public "I told you so's" only rubbed salt into the wound.

VI. Scale Up and Commercialization

Ultimately, the successful transformation of penicillin production into an industrial process required the cooperation of a large number of scientists, private companies, and government agencies. It was in Peoria that the groundwork for a system for mass production was developed. An improved medium for growing the fungus was concocted, containing lactose and corn steep liquor (the fluid left over from the process of making cornstarch). Growth in shallow pans was replaced with a deep fermentation method, derived from brewery techniques, using submerged cultures in large vats. Fleming's penicillin-producing isolate was retired in favor of a new higher yielding strain of *Penicillium chrysogenum* subcultured from a moldy cantaloupe obtained in a local market. Mary Hunt, the woman who found the high-yielding isolate, earned the nickname "Moldy Mary" for her zeal in seeking new strains.

A major interdisciplinary effort was required to develop penicillin. The wartime era fostered an atmosphere of urgency. Pharmaceutical companies broke out of the paradigm that drugs were made synthetically by chemists, and invested in the fermentation equipment that turned filamentous fungi into living factories (Strohl *et al.,* 2001). It is impossible to form an exact chronology of all the specific events in the development of the techniques for industrial-scale production of penicillin. In an unprecedented display of war-driven cooperation, American drug companies joined together with government agencies, private hospitals, and numerous individual scientists, physicians, and

administrators. All of this forms the basis for another story, which is well told in Gladys Hobby's book, *Penicillin: Meeting the Challenge* (Hobby, 1985). Often forgotten, especially in the USA, is the fact that Howard Florey created the spark for this explosion of applied pharmaceutical research, and that after World War II, there was considerable resentment in Britain over the American "theft" of a British discovery (Budd, 1998).

VII. Fleming's Transformation into Celebrity Scientist

In the early days, in both Britain and the USA, penicillin received sporadic press coverage. However, the firestorm that turned Alexander Fleming into a folk hero started with an article published on August 27, 1942 in *The Times* (London). Here is the background: Robert Fleming (Alexander's brother) had an associate named Harry Lambert, who was dying of streptococcal meningitis. Alexander Fleming contacted Florey and asked for some penicillin to treat Lambert. Florey generously gave Fleming almost his entire supply. Fleming treated Mr. Lambert with an intrathecal injection of penicillin. Penicillin had never before been injected into a spinal cord. Lambert recovered, and news that a man had been saved from almost certain death lead to a major article in *The Times*. The August 27th article focused on penicillin and named Oxford as the source of the drug; neither Fleming nor Florey was cited by name. The next day, Sir Almroth Wright wrote a letter to *The Times*, drawing attention to St. Mary's and Fleming.

> Sir: In the leading article on penicillin in your issue yesterday you refrained from putting the laurel wreath for this discovery round anyone's brow. I would, with your permission, supplement your article by pointing out that, on the principle of *palman qui meruit ferat* it should be decreed to Professor Alexander Fleming of this laboratory. For he is the discoverer of penicillin and was the author also of the original suggestion that this substance might prove to have important applications in medicine. (cited in Maurois, 1959, p. 182)

Reporters soon descended on St. Mary's to interview Fleming and several laudatory articles appeared over the next few days. Meanwhile, Sir Robert Robinson, a prominent Oxford chemist, wrote to *The Times* on September 1st, saying that if Fleming deserved a laurel wreath, "A bouquet at least, and a handsome one, should be presented to Professor H. W. Florey." The reporters now descended on Florey. But Florey refused to see them, not only telling his secretary to send them away without any information but also forbidding the entire Oxford group to give any interviews. The inevitable happened. Media coverage focused

on Fleming and St. Mary's; exaggerations and distortions were compounded with omissions and falsifications; the role of the Oxford group was slighted or ignored; in the eyes of much of the public, Alexander Fleming was the sole "inventor" of the greatest life saving drug ever found. Although records show that Fleming never claimed more than he had done, these disclaimers were often ignored by journalists, and Fleming did little to counteract journalistic excesses. Not surprisingly, the Oxford group grew to resent Fleming, even to the point of suggesting that he had engineered his way into the public eye (Clark, 1985; Budd, 1998).

In 1945, Fleming, Florey, and Chain shared the Nobel Prize in Medicine or Physiology. All three men went on to receive numerous other awards, prizes, invited lectureships, and so forth, but it was Fleming who captured the public imagination, Fleming who received the lion's share of popular adulation, and Fleming who is best remembered today. Why?

VIII. Personalities

If temperament and personality are important but intangible parameters in the way in which scientists choose problems and conduct their research, the factors that determine reputation and fame are even more elusive. One reason for the enduring fascination of the penicillin saga is the contrasting personalities of the principal players. Fleming was by all accounts a man "too economical with words," "inarticulate"—afflicted with an "almost pathological lack of conversation." One of Fleming's best friends was stone deaf. Another acquaintance said, "Trying to converse with Fleming was like playing tennis with a man, who when he received a service, put the ball in his pocket." Ronald Hare wrote of "Fleming's inability to express himself clearly and lucidly in either words or print." Paine described Fleming as being a "shocking lecturer, the worst you could possibly imagine" (see Ludovici, 1952; Macfarlane, 1984; Wainright, 1990). Yet there was a paradoxical quality to Fleming's legendary taciturnity. In spite of his silence, "Little Flem" was quite gregarious, had numerous friends, an active social life, and two extremely happy marriages. As a youth, academic success came easily to him; as an adult, he rarely worked more than an eight-hour day. He loved practical jokes and competitive games (shooting, swimming, golf, snooker). His playfulness extended to the laboratory where he took delight in creating "germ paintings" using pigmented bacteria. Examples of this microbial "art" were used as the end papers in the biography by Andre Maurois (1959). After Fleming received the Nobel Prize, he created fungal novelties—medallions of *Penicillium* pressed between

glass. These were presented as gifts to various dignitaries, including members of the British Royal family, the Pope, President Roosevelt, and even Marlena Dietrich. One of these sold for £20,000 ($ 35,160) at Southeby's in 1996 to Pfizer Corporation. Another is in the American Society for Microbiology Archives, a gift from the late Kenneth Raper (Reese, 1996; Brown and Eveleigh, 1997).

Fleming became exceedingly famous, with an intuitive talent at managing the publicity that enveloped him. In turn, the press projected an image of him as an unpretentious and rather mischievous man. He was showered with honorary degrees, prizes, and so forth which he accepted with dignity and no apparent swelling of the ego. Becoming that rarity—the scientific superstar—Alexander Fleming had a wonderful time as a world traveler and celebrity (Figs. 2 and 3).

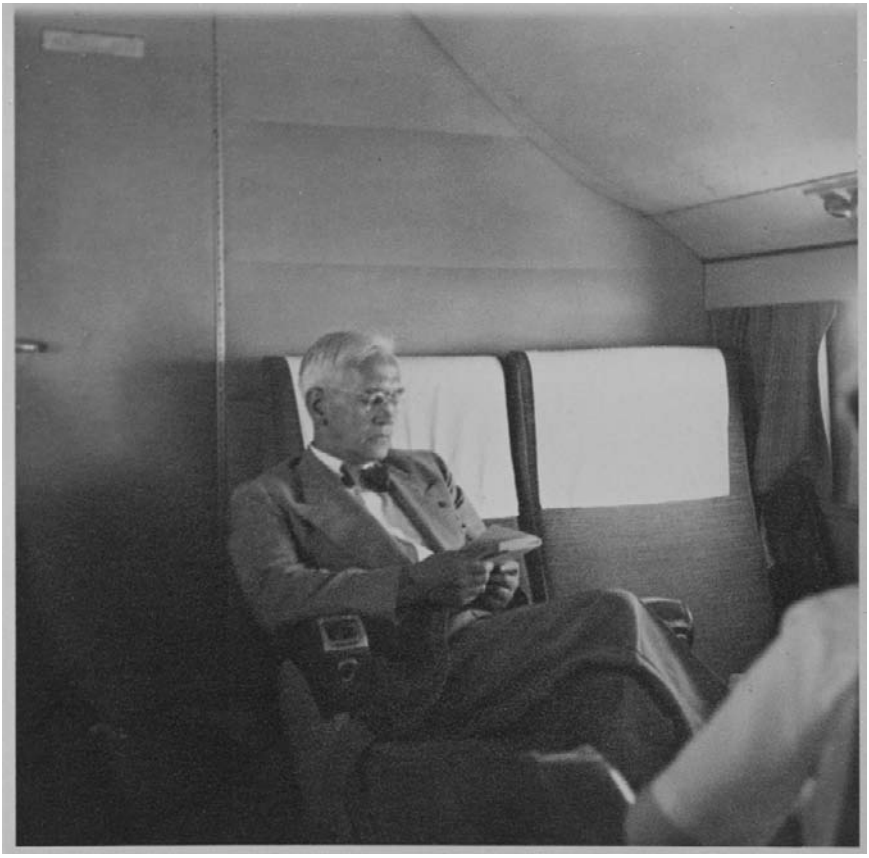

FIG. 2. Alexander Fleming as a world traveler (from the ASM Archives).

FIG. 3. Fleming with Glady Hobby (from the ASM Archives).

Florey never captured the public imagination in the same way, although he came from a more cosmopolitan background than Fleming. Born into modest circumstances in South Australia, he was a Rhodes Scholar who subsequently worked at several important English universities, and befriended many members of the British "Establishment."

Compared to Fleming, he was more visionary, more tenacious, and more driven. He was also a better organizer and manager. In his youth, he won numerous academic awards, but one of his biographers commented that his "dedication to success was seen as a little too intense" (Macfarlane, 1979). Florey had an elegant intellect and unusual determination, but he had difficulty in having fun. Perhaps most important to the unfolding of the penicillin story was his refusal to meet with reporters. Later, he resented how often his group was ignored in the publicity surrounding the new wonder drug, and became bitter at the way in which Fleming was given almost all of the credit (Macfarlane, 1979). Despite Florey's genius, he completely misunderstood the power of the press.

Both Fleming and Florey were reticent; Chain was the opposite. He was flamboyant, emotional, and opinionated. Ronald Clark's biography of Chain is filled with polite phrases about his "extravagant expressions of opinion," "the sometimes counterproductive effects of his own enthusiasm," and his being prone "to not always helpful overstatement." Chain spoke scornfully of the fact that Fleming had not thought of the mouse experiment, and he labeled the Andre Maurois biography of Fleming "a novel." When Chain left England in 1948 to head up an institute in Rome, he stated that one reason for his departure was that "Florey's behavior to me in the years 1941 until October 1948 . . . was unpardonably bad." In later life he was not hesitant to express his disapproval of evolutionary theory ("I would rather believe in fairies than in such wild speculation"); the United Nations ("a completely useless and anachronistic organization"); and the promises of genetic engineering ("more science fiction than science . . . which had not the slightest chance of success."). And as mentioned earlier, when British firms discovered that they might have to pay royalties to manufacture penicillin because of patents held by American individuals and companies, Chain regularly pointed out that he had tried—and been refused permission—to seek similar patents (Clark, 1985).

The media amplified the public pronouncements and personal reputations of the principal players. The open animosity between Florey and Chain, and later the open resentment of the Oxford Group towards Fleming's celebrity, did not help the Oxford cause and added to Fleming's good name. To use a clumsy and nonalliterative contemporary analogy, just in the way late Princess Diana was dubbed "the people's princess," Alexander Fleming became "the people's microbiologist."

IX. Myths and Microbiologists

Over fifty years have passed since the heyday of penicillin research. Life-saving antibiotics are an accepted fact of contemporary life.

Fleming, Florey, and Chain are all dead. Over the decades, journalists, historians, and biographers have written extensively about the people and events surrounding the development of penicillin, often with the intent of debunking Fleming's role. Nevertheless, when all is said and done, we find that Fleming remains a hero, not just in the public imagination but also among scientists. Why his continuing fame? What is it about Alexander Fleming and the penicillin story that continues to attract our attention?

Perhaps part of the reason we like the Fleming story is because he was such an unlikely superhero. Inarticulate and unassuming, he did not seem destined for glory. Scientists of the Oxford University, most of whom were far more adept with words, who came from wealthier backgrounds, who studied at "better" universities, and who worked at more prestigious institutions than St. Mary's, seemed more probable candidates for public adoration. Any scientist who has ever felt the subtle sting of scientific snobbery can empathize with Fleming's sweet success. Moreover, Fleming's success did not come early. His election to the Royal Society, his knighthood, and his Nobel Prize all were awarded when he was well over sixty years of age.

Fleming also appeals to us in other ways. He always labeled himself as a simple bacteriologist, downplaying the fact that he was a physician. And he was a "bacteriologist's bacteriologist," most comfortable working with his hands at the bench, a keen observer and superb experimentalist, preferring to use simple, classical equipment (see Fig. 4 for a reproduction of his laboratory bench).

Finally, by all reports, fame did not spoil Fleming. He remained down-to-earth and unpretentious. When others grew critical of his fame, Fleming was forced to defend himself on more than one occasion. A flavor of the man can be derived from this version of his response, as recorded in the biography by Ludovoci:

> At St. Mary's I had been short of chemical help, and at the School of Hygiene Raistrick had lacked complete bacteriological co-operation. Because of the lack of some co-ordinating standard institute, there was a gap of nearly ten years before chemists, bacteriologists and others got together at Oxford and concentrated penicillin sufficiently to show its remarkable curative properties in infective disease. Even then facilities for development were lacking, and the information was taken to America where certain developments took place, so that America has justly reaped a large part of the reward. Had we a central institute, where a complete team of workers could have developed penicillin, this one discovery might well have paid for the institute ... to say nothing of the suffering that might have been avoided in the ten years incubation period .... Nevertheless, I venture to suggest that in medicine we will never initiate anything by teamwork.

FIG. 4. Laboratory bench at St. Mary's Hospital (from St. Mary's Archives).

> That comes later. There is need for a continuation of individual enterprise in research. I was working on another project entirely when I observed the unusual effects of penicillin. So I abandoned my first project and devoted myself to the new discovery. Had we been working as a team, that first oddity would have been thrown away (Ludovici, 1952, pp. 151–52).

As scientists, most of us believe passionately in "individual enterprise in research," and we recognize that the gift for new discovery can occur in unlikely places. Teamwork brought penicillin out of the laboratory, but an individual made the discovery on which the teamwork was based. Fleming exemplifies our belief in solo scientific breakthroughs.

In reviewing twentieth-century microbiology, we could do far worse than to honor the memory of Alexander Fleming: his experimental proficiency, his reticence, his media savvy, and his playfulness. Although luck played a big part in gaining his place in the scientific pantheon, he wore his renown with such an unaffected grace that those who criticized him were somehow diminished.

Practicing scientists know that the course of science does not run smoothly. In the long run we can decide only in retrospect which discoveries are important and which are not. In retrospect, Alexander Fleming's discovery grows in significance. Penicillin is the paradigm for natural products drug discovery; Fleming himself is an enduring role model not only for success in clinical research but also for how to project a positive popular image for the scientific profession.

ACKNOWLEDGMENTS

Ronald Bentley (University of Pittsburgh), and Jeff Karr (ASM Archives) reviewed a manuscript version of this paper, and made many improvements in content and style. We are grateful for their generous scholarly input. We also thank Douglas Eveleigh (Rutgers University) for his useful comments, and Jason Beadle for help with manuscript preparation.

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