

Marine *Bacillus* Spores as Catalysts for Oxidative Precipitation and Sorption of Metals

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Abstract

The oxidation of soluble manganese(II) to insoluble Mn(III,IV) oxide precipitates plays an important role in the environment. These Mn oxides are known to oxidize numerous organic and inorganic compounds, scavenge a variety of other metals on their highly charged surfaces, and serve as electron acceptors for anaerobic respiration. Although the oxidation of Mn(II) in most environments is believed to be bacterially-mediated, the underlying mechanisms of catalysis are not well understood. In recent years, however, the application of molecular biological approaches has provided new insights into these mechanisms. Genes involved in Mn oxidation were first identified in our model organism, the marine *Bacillus* sp. strain SG-1, and subsequently have been identified in two other phylogenetically distinct organisms, *Leptothrix discophora* and *Pseudomonas putida*. In all three cases, enzymes related to multicopper oxidases appear to be involved, suggesting that copper may play a universal role in Mn(II) oxidation. In addition to catalyzing an environmentally important process, organisms capable of Mn(II) oxidation are potential candidates for the removal, detoxification, and recovery of metals from the environment. The Mn(II)-oxidizing spores of the marine *Bacillus* sp. strain SG-1 show particular promise, due to their inherent physically tough nature and unique capacity to bind and oxidatively precipitate metals without having to sustain growth.

Introduction

Microorganisms capable of manganese(II) oxidation have been recognized since the beginning of the 20th century (Jackson, 1901) but, even today, the underlying mechanisms and biological function of this process remain poorly understood. Despite a century of isolating and characterizing an amazing diversity of Mn(II)-oxidizing bacteria from a wide variety of environments, only recently has significant progress been made towards elucidating the mechanisms for enzymatic Mn(II) oxidation. This progress has been due primarily to the application of

molecular and biochemical approaches to the study of bacterial Mn(II) oxidation.

The primary focus of this article is to review our current view of the mechanism for Mn(II) oxidation of the marine *Bacillus* sp. strain SG-1, with particular emphasis on the molecular genetic and biochemical aspects. In addition, comparisons with two other model bacterial Mn(II)-oxidation systems allow us to speculate regarding a more universal mechanism of Mn(II) oxidation. Finally, we review the unique metal binding and oxidation properties of SG-1 spores which make them attractive candidates for biotechnological applications, such as the bioremediation of metal pollution.

Background on Manganese(II) Oxidation

General Chemistry of Manganese

Manganese (Mn) is an essential nutrient for all living organisms, serving as a cofactor in a variety of enzymes (Larson and Pecoraro, 1992), including superoxide dismutase and the active site of photosystem II. Manganese is the second most abundant transition metal, behind iron, in the earth's crust and the fifth most abundant metal on the surface of the earth. Although Mn can occur in oxidation states ranging from 0 to +7, the +2, +3, and +4 oxidation states are most relevant under natural environmental conditions. In nature, Mn is generally found as reduced soluble or adsorbed Mn(II) and as highly insoluble Mn(III) and Mn(IV) oxides and oxyhydroxides, which appear as brownish-black precipitates. Mn(IV) minerals are ultimately the most thermodynamically stable form in nature.

Abiotic Mn Oxidation

The oxidation of soluble Mn(II) to Mn(III,IV) oxides is a thermodynamically favorable, but kinetically slow, reaction at neutral pH. Because of this, Mn(II) oxidation in natural systems, such as groundwater and surface waters, often proceeds at very slow rates in the absence of bacteria (Diem and Stumm, 1984; Nealson *et al.*, 1988). Abiotic chemical oxidation of Mn(II) generally only occurs under extreme conditions within a few weeks to months. In marine environments, soluble Mn can vary between 10^{-9} M in seawater to 10^{-4} M in pore waters of some sediments (Rosson and Nealson, 1982). Mn(II) oxidation is autocatalytic, with the Mn(oxyhydr)oxide products adsorbing Mn(II) and catalyzing its further oxidation. In addition, a variety of other surfaces like Fe oxides and silicates also catalyze Mn(II) oxidation. Mn oxides play an important role in the marine environment, where they are known to oxidize a number of organic and inorganic compounds, serve as electron acceptors for anaerobic bacteria, and scavenge many other metals (*e.g.*, Cu, Co, Cd, Ni, and Zn) on their highly charged surfaces.

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Biological Mn Oxidation

Although the production of Mn oxides in most environments is considered to be predominantly microbially mediated (Nealson *et al.*, 1988), the mechanisms of catalysis (and biological function) are poorly understood. Mn(II)-oxidizing organisms are widely distributed in nature and occur wherever soluble Mn(II) species occur, from marine and freshwaters, to sediments, soils, and desert varnish (Ghiorse, 1984). Certain algae, yeast, and fungi have been shown to catalyze Mn(II) oxidation, but bacteria are believed to be the most important Mn(II)-oxidizing organisms in aquatic environments (Tebo *et al.*, 1997; Tebo, 1998). As a group, Mn(II)-oxidizing bacteria are phylogenetically diverse. Based on 16S rRNA sequencing, all Mn(II)-oxidizers analyzed to date have fallen within the Gram-positive or *Proteobacteria* branches of the Domain Bacteria (Tebo *et al.*, 1997). In addition, all of the Gram-negative organisms have fallen within the α , β , and γ *Proteobacteria*.

There are two general mechanisms of Mn oxidation (Nealson *et al.*, 1989) which can be operationally described as indirect or direct. Indirect oxidation may occur via an increase in pH or E_h , while direct oxidation generally occurs via the active binding and oxidation of Mn(II) by an enzyme. Mn(II)-oxidizing activity has been reported in cell-free extracts of many bacteria (Ehrlich, 1968; Douka, 1977; Jung and Schweissfurth, 1979; Douka, 1980), but the specific Mn-oxidizing components have only been characterized in a few cases, namely: *Leptothrix discophora*, *Pseudomonas putida*, and our model organism, the marine *Bacillus* sp. strain SG-1.

Leptothrix sp. are sheath-forming organisms which are ubiquitous in wetlands, iron seeps, and springs around the world. *L. discophora* is characterized by the precipitation of both iron and manganese oxides on its sheaths. The sheathless mutant strain SS-1 excretes a manganese-oxidizing factor, normally associated with the sheath, into the culture medium. This ~110 kDa protein, designated MofA, is capable of forming a Mn oxide band in SDS-PAGE gels incubated in $MnCl_2$. MofA was the first Mn-oxidizing protein to be purified and partially characterized (Adams and Ghiorse, 1987; Boogerd and deVrind, 1987). The oxidizing activity is inhibited by cyanide, azide, *o*-phenanthroline, mercuric chloride, and pronase. The gene *mofA*, encoding the putative Mn(II)-oxidizing protein of SS-1, was recently cloned and sequenced (Corstjens *et al.*, 1997), which revealed that the encoded protein sequence shares significant similarity with multicopper oxidases (see below). However, further analysis of the molecular mechanism of Mn oxidation of *L. discophora* has been hampered by the current lack of genetic tools for use in these organisms.

Pseudomonas putida is a ubiquitous freshwater and soil bacterium and, thus, provides an excellent model system for studying bacterial Mn oxidation. The closely related strains MnB1 and GB-1 have been intensively studied in recent years. Upon reaching stationary phase, these organisms oxidize Mn(II) to Mn(IV) oxyhydroxides which are precipitated on the cell surface. Previous studies demonstrated that MnB1 produces a soluble Mn(II)-oxidizing protein late in logarithmic phase (Jung and Schweissfurth, 1979; DePalma, 1993). More recent biochemical studies with GB-1 resulted in the partial purification and characterization of two Mn(II)-oxidizing factors with estimated molecular weights of 180 kDa and

250 kDa (Okazaki *et al.*, 1997). The Mn-oxidizing activity of these factors is sensitive to azide and mercuric chloride, and inhibited by cyanide, EDTA, Tris, and *o*-phenanthroline. Unlike MofA of *L. discophora*, the Mn(II)-oxidizing factors of GB-1 are more sensitive to SDS and only produce Mn oxide bands in native polyacrylamide gels (lacking SDS). Rather than the existence of two distinct Mn-oxidizing proteins, it is more likely that the Mn-oxidizing protein(s) isolated are part of a larger complex which degrades into smaller fragments that retain activity (Okazaki *et al.*, 1997).

In contrast to *L. discophora*, there are a variety of well-developed genetic tools available for molecular genetic analysis of *Pseudomonas* species. Recent studies have used transposon mutagenesis to identify genes involved in Mn oxidation in both *P. putida* strain MnB1 and GB-1 (Caspi *et al.*, 1998; de Vrind *et al.*, 1998). In both studies, genes involved in the biogenesis and maturation of *c*-type cytochromes were found to be essential for Mn oxidation. However, cytochromes alone are not thought to be sufficient for catalyzing the oxidation of manganese. In GB-1, a gene encoding a multicopper oxidase, designated *cumA*, was found to be essential for Mn-oxidation (Brouwers *et al.*, 1999). In addition, small amounts of Cu^{2+} were found to increase the Mn(II)-oxidizing activity of wild-type cells by a factor of 5. Thus, it has been proposed that this Cu-dependent oxidase is an important constituent of the oxidizing complex and may directly oxidize Mn(II). The importance of copper in the mechanism of bacterial manganese oxidation will be further discussed later in this review.

Marine *Bacillus* sp. Strain SG-1

General Properties

Spore-forming *Bacillus* species can be a significant component of the total colony-forming bacteria in certain aquatic environments (20 to 40%) and sediments (up to 80%) (Bonde, 1981). Within the genus *Bacillus*, a variety of organisms are known to oxidize Mn(II). Some oxidize Mn(II) during vegetative growth (Ehrlich and Zapkin, 1985; Ehrlich, 1996) or only during the onset of sporulation (Vojak *et al.*, 1984), but there is a major group, at least in marine environments, that produce mature spores that oxidize Mn(II) (Lee, 1994). In fact, a considerable portion (17-33%) of the spore-forming bacteria isolated from coastal surface sediments of Mission Bay and San Diego Bay, California, were found to produce Mn(II)-oxidizing spores (Lee and Tebo, unpublished).

The marine *Bacillus* sp. strain SG-1 was isolated from a Mn-coated sand grain that was obtained from a shallow marine sediment off Scripps pier (Nealson and Ford, 1980). This organism produces metabolically dormant spores that bind and oxidize Mn(II), thereby becoming encrusted with Mn oxide (Figure 1). SG-1 spores are also capable of binding a variety of other heavy metals such as copper, cadmium, zinc, nickel, and cobalt (the latter of which is also oxidized) (Tebo and Lee, 1993; Lee and Tebo, 1994; Tebo, 1995). The vegetative cells of SG-1, on the other hand, do not oxidize Mn and have actually been shown to reduce Mn oxide under oxygen limiting conditions (de Vrind *et al.*, 1986a). This suggests that one possible purpose of Mn oxidation by these spores is to store up Mn oxides as an electron acceptor for growth under low oxygen or anaerobic conditions, upon germination in the sediments (Tebo, 1983; de Vrind *et al.*, 1986a).

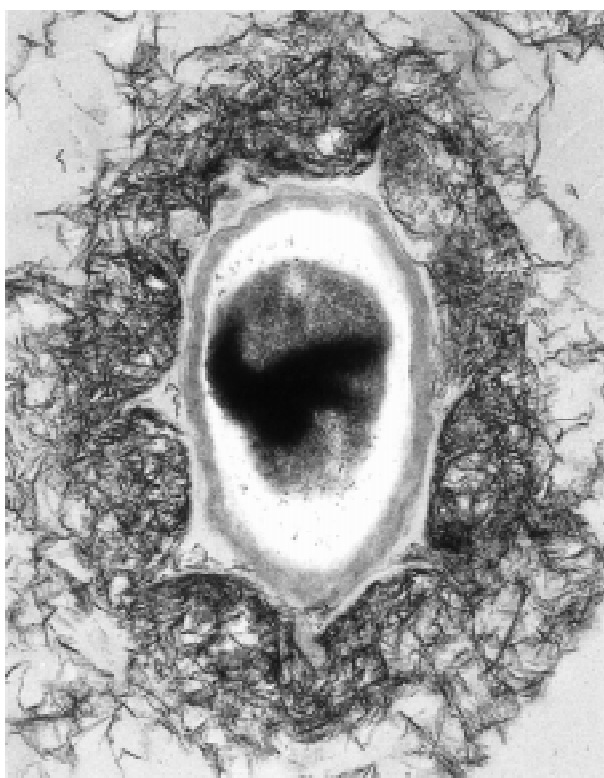


Figure 1. Spores of *Bacillus* sp. Strain SG-1
Transmission electron micrograph of a thin section of the metal-oxidizing spores of the marine *Bacillus* sp. strain SG-1. The spores are coated with manganese oxides. Approximate spore size: 1.25 x 0.75 μ m.

Biochemistry

Manganese oxidation by SG-1 spores occurs over a wide range of environmental conditions including: metal concentration (<nM to >mM), temperature (<3 °C to > 70 °C), pH (>6.5), and osmotic strength (from distilled water to seawater) (Rosson and Nealon, 1982). In fact, the spores can even be rendered non-germinable with glutaraldehyde, formaldehyde, or UV light, and still retain Mn oxidizing activity (Rosson and Nealon, 1982). The oxidizing activity of the spores is heat labile and is poisoned by the metalloprotein inhibitors azide, cyanide, and mercuric chloride (Rosson and Nealon, 1982). Transmission electron microscopy demonstrated that the Mn oxide is precipitated on the ridged outermost spore layer (Tebo, 1983). Spore coat preparations, processed to retain all the outer layers and remove the spore contents, were shown

to retain full oxidizing activity (de Vrind *et al.*, 1986b). These results suggested that a protein component of the outermost spore layer, either the spore coat or exosporium, is responsible for catalyzing the oxidation of manganese.

The spore coat is a highly cross-linked structure which gives the spore resistance to chemical attack and mechanical disruption (Warth, 1978; Driks, 1999). An additional layer found in some, but not all, spores is termed the exosporium. The exosporium is a loose-fitting outermost layer composed of protein, lipid, and carbohydrate, and has no known function (Matz *et al.*, 1970; Tipper and Gauthier, 1972). Although this layer has been hypothesized to play a protective role, this is somewhat controversial since it is not found in spores of all species. Unlike the spore coat, there is very little information available regarding the exosporium at the genetic, biochemical, or developmental level. Recent studies in our laboratory suggest that the Mn(II)-oxidizing activity of SG-1 spores is localized to an exosporium (Francis *et al.*, 1997).

Over the years, attempts have been made to isolate the Mn(II)-oxidizing protein(s) by extracting proteins from SG-1 spores, separating them by SDS-PAGE, and incubating the gels with Mn(II) (Tebo *et al.*, 1988). A high molecular weight Mn-oxidizing band (~205 kDa) has occasionally been observed in gels. Re-extraction of this band, followed by SDS-PAGE, and Coomassie staining revealed that it was composed of several proteins. However, these experiments are difficult to reproduce, from experiment to experiment, possibly due to damaging of the Mn-oxidizing factors during extraction, or because several components that are separated during electrophoresis may be required for activity.

Genetics

Due to the difficulties in consistently recovering Mn(II)-oxidizing activity from spores for biochemical studies, our laboratory employed a molecular genetic approach to study Mn oxidation by SG-1. Methods for plasmid transformation and transposon mutagenesis were developed for SG-1 (van Waasbergen *et al.*, 1993). Using the temperature sensitive plasmid pLTV1, which carries Tn917, a promoterless *lacZ* gene, and an *Escherichia coli* replicon, 27 independent non-oxidizing, but still sporulating, mutants were isolated. Out of the 27 mutants, 18 of the insertions turned out to map within a contiguous cluster of seven genes, the *mnx* genes (van Waasbergen *et al.*, 1996). This work was the first report to identify genes involved in Mn oxidation, as well as the first to describe a genetic system developed for a marine Gram-positive bacterium.

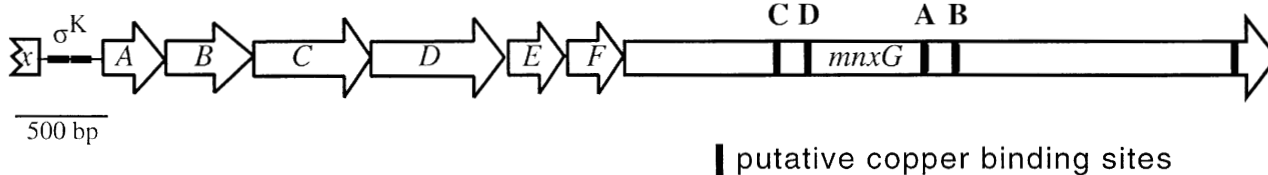


Figure 2. The *mnx* Gene Cluster

The organization of the *mnx* gene cluster of the marine *Bacillus* sp. strain SG-1 based on DNA sequence analysis. *mnxG* encodes the putative Mn(II)-oxidizing protein which shares significant similarity with multicopper oxidases, particularly in the regions of copper binding (boxed areas). The amino acid sequences of the copper binding sites designated with the letters A-D are shown in Figure 3. σ^K represents the putative -35 and -10 consensus promoter sequences which precede this operon.

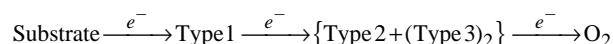
The *mnx* gene cluster appears to be organized in an operon (Figure 2) which is preceded by a potential recognition site for the sporulation, mother-cell-specific, RNA polymerase sigma factor, σ^K . Consistent with this, measurement of β -galactosidase activity from a Tn917-*lacZ* insertion in *mnxD* showed expression at mid- to late sporulation (approximately stage IV to V of sporulation). Spores of nonoxidizing mutants appeared unaffected with respect to their temperature and chemical resistance properties as well as germination characteristics. However, in some of the mutants, transmission electron microscopy revealed slight alterations in the ridged outermost spore layer, consistent with the localization of Mn(II)-oxidizing activity to this layer.

Possible Mechanism of Mn Oxidation

Sequence analysis of the *mnx* gene cluster revealed that three of the encoded proteins (MnxA, MnxB, and MnxE) were predicted to be highly hydrophobic, while only two of the proteins (MnxC and MnxG) showed significant similarity to other proteins in the databases. MnxG is a predicted 138 kDa protein which shows similarity to the family of multicopper oxidases (Figure 3), a diverse group of proteins that utilize multiple copper ions as cofactors in the oxidation of a variety of substrates (Ryden and Hunt, 1993). Members of this family include ascorbate oxidase (from squash and

cucumber), laccase (from plants and fungi), ceruloplasmin (from vertebrates), FET3 (from yeast), and CopA (a copper resistance protein from *Pseudomonas syringae*). Of these proteins, only ceruloplasmin and FET3 are known to oxidize a metal, Fe(II), as a substrate.

Multicopper oxidases are a unique class of enzymes which can be defined by their spectroscopy, sequence homology, and reactivity (Solomon, 1996). All multicopper oxidases contain copper ions of three spectroscopically distinct types ('blue' copper (or Type 1), Type 2, and Type 3) with the minimum functional unit containing at least one Type 1 site and a Type 2/Type 3 trinuclear cluster. The amino acids (histidine, cysteine, and methionine/leucine/phenylalanine) which make up each copper center come into close proximity to one another and coordinate copper. The Type 1 center accepts the initial electron from the substrate and shuttles it to the Type 2/Type 3 center which binds and reduces molecular oxygen:



Only multicopper oxidases and cytochrome oxidases are known to couple the four electron reduction of O_2 to H_2O with the oxidation of substrate. In the well-characterized multicopper oxidases, the substrate is oxidized by one

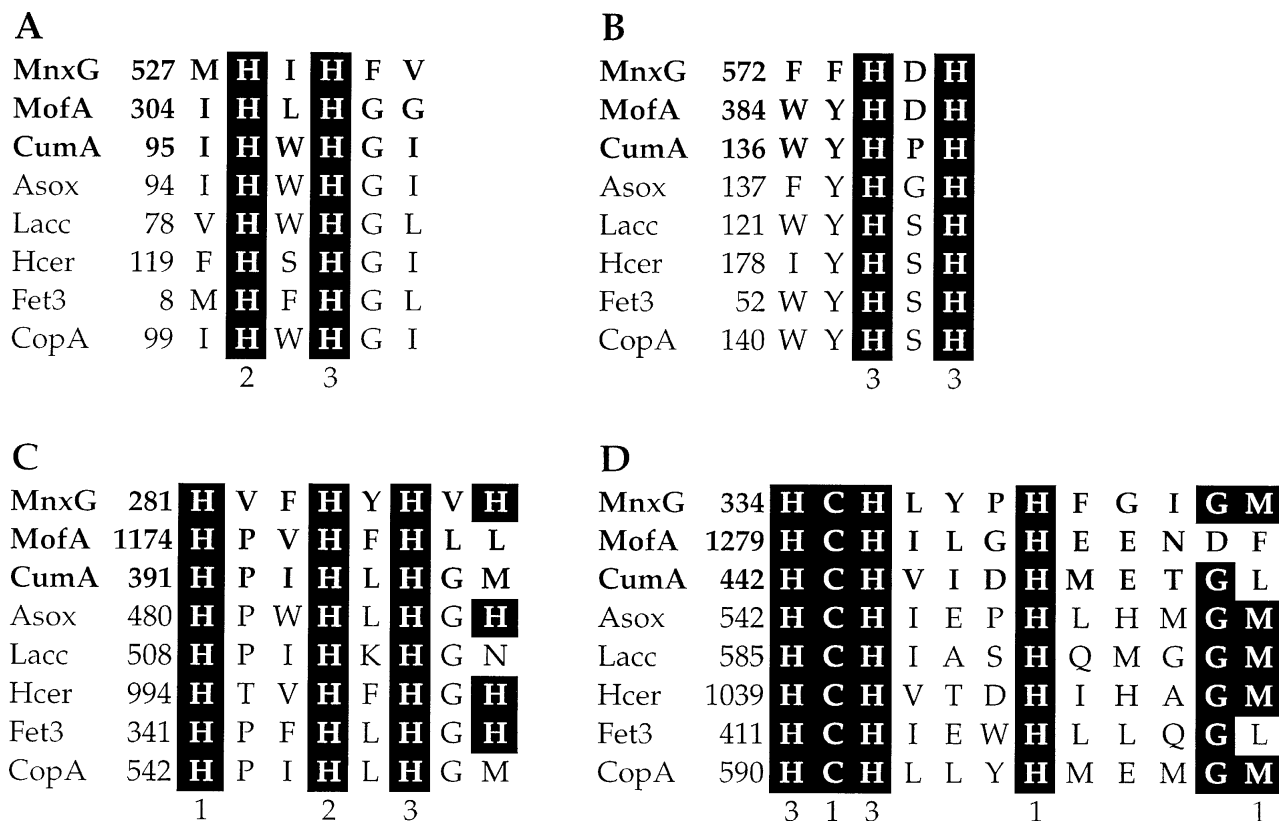


Figure 3. Copper-binding Sites in Multicopper Oxidases

Amino acid alignment of the copper-binding sites in MnxG, MofA, CumA, and other multicopper oxidases. The letters A-D correspond to the copper binding sites shown in Figure 2. Abbreviations: Asox, ascorbate oxidase (cucumber and squash); Lacc, laccase (fungi); Hcer, human ceruloplasmin; FET3, an iron oxidizing/transport protein in yeast; and CopA, a copper-resistance protein from *Pseudomonas syringae*. The amino acids conserved among the different proteins are shaded and the copper-binding residues are numbered according to the spectroscopic type of copper they potentially help coordinate.

electron. Thus, if Mn(II) oxidation is, indeed, catalyzed by a multicopper oxidase, it is most likely that Mn(II) is oxidized by sequential one electron transfers in which Mn(III) is a transient intermediate.

Both X-ray crystallography and comparative sequence analysis have demonstrated that multicopper oxidases possess a distinctive subdomain structure (Solomon *et al.*, 1996). Laccase, ascorbate oxidase, and FET3, all appear to have three domains while, the larger enzyme, ceruloplasmin has six domains. These copper enzymes all exhibit significant internal homology among the subdomains, suggesting that they all arose from a common ancestor by gene duplication (Ryden and Hunt, 1993; Solomon *et al.*, 1996).

MnxG shares significant similarity with the multicopper oxidases, particularly in regions surrounding the conserved copper binding regions. Based on size and subdomain structure, MnxG appears to be most similar to the Fe(II)-oxidizing protein, ceruloplasmin, containing six subdomains. Azide, a potent inhibitor of multicopper oxidases that acts by bridging the Type 2 and Type 3 copper atoms, has also been found to inhibit Mn(II) oxidation by SG-1 spores. Conversely, small amounts of copper actually enhance the rate of Mn(II) oxidation by the spores (van Waasbergen *et al.*, 1996). The sequence similarity of MnxG to multicopper oxidases, combined with the copper-enhancement and azide-inhibition of Mn(II) oxidation, suggests that MnxG may function like a copper oxidase and directly oxidize manganese.

Although MnxG may be the only Mnx protein directly involved in Mn oxidation, it is possible that one or more of the other Mnx proteins may also be required for activity. In particular, MnxC shares significant similarity with several proteins involved in multicomponent oxidoreductase systems, suggesting that MnxC and MnxG might also part of such a system. MnxC is a predicted 22 kDa protein that has a putative N-terminal signal sequence, indicating that it may be associated with a membrane. It shares sequence similarity with a number of cell surface and multicomponent oxidoreductase-associated proteins which all share a C-XXX-C motif. One of these proteins, an 18 kDa protein in the mercury resistance operon of *Staphylococcus aureus* (Laddaga *et al.*, 1987), has a thioredoxin motif surrounding these cysteine residues (C-XX-C), suggesting that these residues may exhibit redox activity and be involved in the formation of disulfide bonds (Ellis *et al.*, 1992). An alternative, and perhaps more intriguing, function for these cysteine residues comes from the similarity of MnxC to two other proteins, SCO1 and SCO2 of *Saccharomyces cerevisiae*. These proteins were previously shown to play an essential role in the assembly of the mitochondrial cytochrome oxidase complex (Schulze and Roedel, 1989). More recently, the two cysteines of SCO1 have been suggested to bind and deliver copper to the copper-containing protein, cytochrome oxidase, thus conferring activity (Glerum *et al.*, 1996). By analogy, MnxC may be involved in delivering copper to the multicopper oxidase, MnxG, giving it activity. Interestingly, the multicopper oxidases, ceruloplasmin and FET3, both require additional proteins to deliver copper to them and, thus, confer oxidase activity (Stearman *et al.*, 1996). A possible association between MnxC and MnxG is supported by the recent localization of both of these proteins to the exosporium of SG-1 spores (Francis and Tebo, unpublished).

Role of Copper in Bacterial Mn Oxidation

The bacterial Mn oxidation systems that have been characterized at the molecular level in recent years all seem to be linked by the apparent use of copper as an essential enzymatic cofactor. Three otherwise unrelated organisms, a *Leptothrix*, a *Pseudomonas*, and a *Bacillus* species, appear to be utilizing enzymes related to multicopper oxidases for the oxidation of manganese. Despite their involvement in catalyzing the same reaction, these extracellular proteins have unique locations within their respective organisms: within an extracellular sheath, an outer membrane, and an outermost spore layer. None of these proteins share strong overall sequence similarity with one another, but they all contain the conserved copper-binding regions always found in multicopper oxidases. In addition to sequence homology, there is also biochemical evidence to support the role of copper in bacterial Mn(II) oxidation. First, the Mn(II)-oxidizing activity of all three of these systems is inhibited by azide, a potent inhibitor of multicopper oxidases. Second, copper has been shown to significantly enhance the rate of Mn(II)-oxidation in both the *Bacillus* sp. strain SG-1 and *P. putida* GB-1 (Brouwers *et al.*, 1999), but has not yet been thoroughly tested in *L. discophora* SS-1. Finally, it has recently been demonstrated that Mn(II) oxidation in yet another phylogenetically distinct organism, the prosthecate bacterium *Pedomicrobium* sp. ACM 3067, also appears to be catalyzed by a copper-dependent enzyme (Larsen *et al.*, 1999). Although the well-known multicopper oxidases have been shown to oxidize a wide variety of substrates, until recently, Fe(II) was the only known metal substrate. Thus, it is possible that bacterial Mn oxidases may constitute a new functional group of multicopper oxidases. However, definitive proof of this hypothesis awaits further biochemical and spectroscopic analysis of these Mn(II)-oxidizing enzymes.

Potential Biotechnological Applications

In addition to providing an excellent model system for studying the molecular and biochemical mechanisms of metal precipitation, SG-1 spores have a number of unique properties that make them attractive candidates for biotechnological applications, such as environmental remediation of metal pollutants (Figure 4).

Recent characterization of the surface chemistry and Cu(II) adsorption properties of SG-1 spores revealed that, in addition to actively binding and oxidizing Mn(II), they also have an extensive capacity for passively binding other metals (He and Tebo, 1998). The specific surface area of the spores was found to be around $74.7 \text{ m}^2\text{g}^{-1}$, a fairly high value in the range similar to metal (hydr)oxides and other clay minerals. Like most bacterial surfaces, the SG-1 spore surface has a net negative charge with a point of zero charge at pH 4.5. The surface was shown to be dominated by negatively charged sites, which are most likely carboxylate but also phosphate groups, consistent with the presence of both protein and carbohydrate in the outermost layer of the spores. Copper adsorption by SG-1 spores is rapid and complete within minutes, with adsorption starting at pH 3 and increasing with pH (Figure 5). The high surface area and surface site density of SG-1 spores is comparable to that of Fe, Mn, and Al mineral colloids, accounting for the fact that these spores have an extensive capacity for binding copper and other toxic metals on their surface

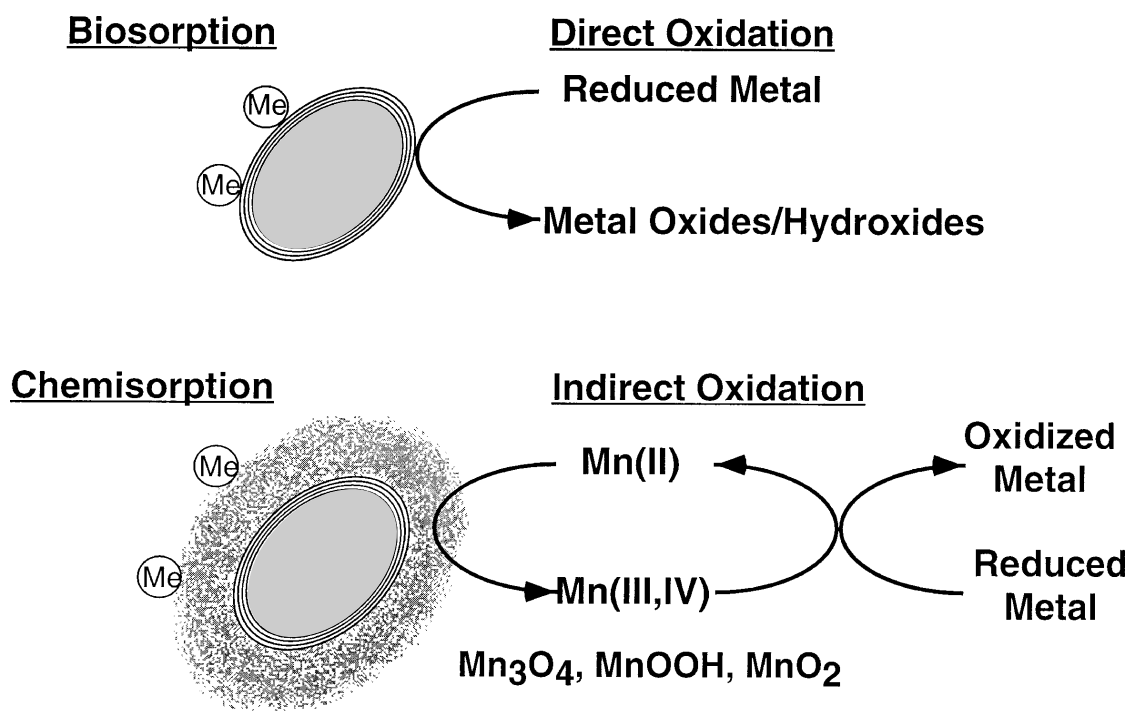


Figure 4. Schematic Representation of the Mechanisms by which SG-1 Spores can Either Adsorb or Oxidize Various Metals
Top: The spores can passively adsorb certain metals (where **Me** = Cu, Cd, Zn, Ni) on the charged spore surface (biosorption). The enzymatic activity of the outermost spore layer can also directly catalyze the oxidation of divalent metals such as Mn(II) and Co(II) (direct oxidation). **Bottom:** The highly charged Mn oxides which form on the spore surface are capable of nonspecifically adsorbing (chemisorption) a variety of metals (where **Me** = Cu, Co, Cd, Zn, Pb, radionuclides, etc.). The Mn oxides are also strong oxidants, capable of indirectly oxidizing many metals and organics (indirect oxidation).

(Figure 6). In fact, SG-1 spores have also been shown to bind both Cd(II) and Zn(II) (Tebo, 1995). The Cu(II) adsorption affinity coefficient (K) and the adsorption capacity (Γ_m) of the spores calculated from the Langmuir equation are $2.08 \times 10^6 \text{ L}\cdot\text{mol}^{-1}$ and $10.77 \mu\text{mol}\cdot\text{m}^{-2}$ respectively. The K value is simply the inverse of the substrate binding constant ($K_s = 0.48 \mu\text{M}$) with which most

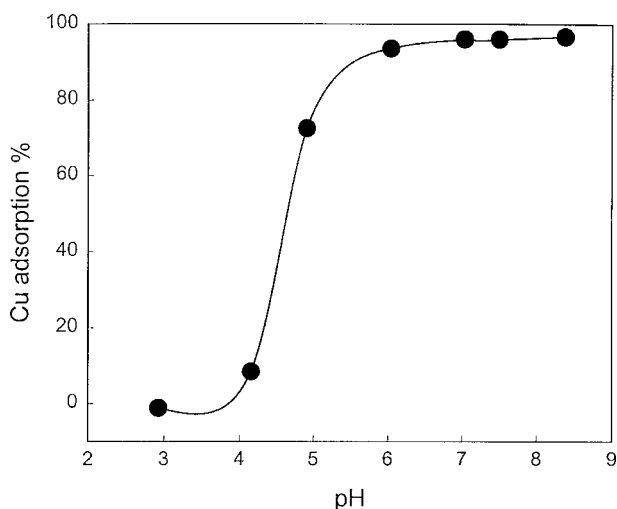


Figure 5. Cu(II) Adsorption by SG-1 Spores
 Cu(II) adsorption by SG-1 spores as a function of pH [10^8 spores ml^{-1} , 0.01 M NaNO_3 , $2 \mu\text{M Cu(II)}$]. The adsorption value for 100% adsorption is $1.5 \mu\text{mol}\cdot\text{m}^{-2}$. Reproduced with permission from He and Tebo (1998).

biologists are familiar. The spore affinity for Cu(II) is 2-4 orders of magnitude greater (*i.e.* the K_s is 2-4 orders of magnitude lower) than the affinities of Cu(II) determined for a variety of other biomasses, including fungi, bacteria and algae, or for an alginate gel (He and Tebo, 1998). The adsorption capacity is on the high end of the range observed for other types of biomass. Thus, in the absence of Mn, SG-1 spores may act as good passive adsorbents for the removal of metals and radionuclides from contaminated waters.

SG-1 spores also have the unique capacity to bind and oxidize cobalt (Tebo and Lee, 1993; Lee and Tebo, 1994), even in the absence of Mn(II) or preformed Mn oxides. Like Mn, the concentrations of Co in the environment rarely reach toxic levels. However, the radionuclide ^{60}Co is an activation product in radioactive wastes and has been identified as a priority pollutant at various Department of Energy sites in the United States. Since the oxidation of Co(II) results in the formation of solid Co(III)(oxy)hydroxide precipitates, the Co binding and oxidizing properties of SG-1 spores may be useful for dealing with ^{60}Co problems. The Co(II)-oxidizing properties of SG-1 spores are similar to those for Mn(II) oxidation, with oxidation occurring over a wide range of pH, temperature, and Co(II) concentrations (Lee and Tebo, 1994). Optimal Co(II) oxidation occurs around pH 8 and at 55° to 60°C . Co(II) can be oxidized at the trace levels found in seawater all the way up to 100 mM, with the oxidation following Michaelis-Menton kinetics. Based on the kinetic studies, it appears that SG-1 spores have two oxidation systems for Co(II), a high-affinity-low rate system ($K_M = 3.3 \times 10^{-8} \text{ M}$; $V_{\text{max}} = 1.7 \times 10^{-15} \text{ M}^{-1}\cdot\text{h}^{-1}$) and a low-

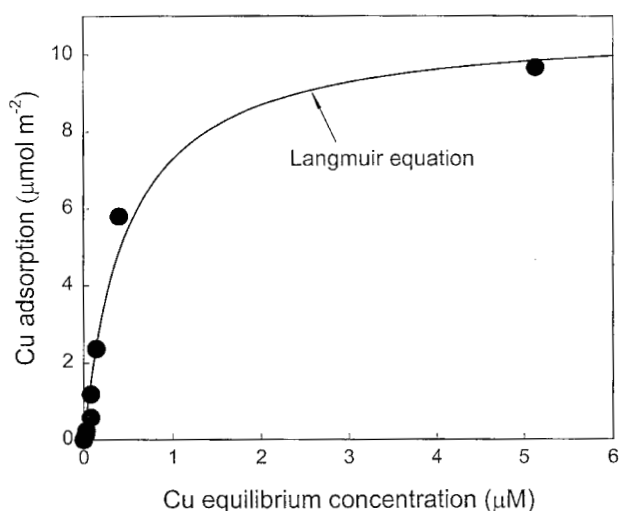


Figure 6. Cu(II) Adsorption Isotherm
Cu(II) adsorption isotherm obtained with SG-1 spores (10^8 spores ml^{-1} , 0.01 M NaNO_3 , pH 7.0). The curve was obtained by fitting the data to the Langmuir equation. The results demonstrate that the spores have both a high affinity ($K_s = 0.48 \mu\text{M}$) and a high adsorption capacity for Cu ($10.77 \mu\text{mol}\cdot\text{m}^{-2}$ of spore surface or $0.83 \text{mmol}\cdot\text{g}^{-1}$ dried spores). Reproduced with permission from He and Tebo (1998).

affinity-high-rate system ($K_M = 5.2 \times 10^{-6} \text{M}$; $V_{\text{max}} = 8.9 \times 10^{-15} \text{M}^{-1}\cdot\text{h}^{-1}$) (Lee and Tebo, 1994). It is likely that both Mn(II) and Co(II) oxidation occur at the same active site, since spores of transposon mutants within the *mnx* gene cluster do not oxidize Mn(II) or Co(II). The K_M for the high-affinity system (33 nM) suggests that SG-1 spores can remove metals (at least Co and Mn) to much lower levels than achieved by other chemical and biological procedures.

Mn oxides have long been recognized for their role in scavenging metals and radionuclides in the environment (Murray, 1975; Hem, 1978). The highly charged surfaces scavenge a variety of trace elements (e.g. Cu, Co, Cd, Zn, Pb) and radionuclides (e.g. ^{210}Pb , ^{60}Co), as well as Ra and Th isotopes, and can lead to the reduction in the concentration of soluble trace metals by several orders of magnitude. Thus, bacteria capable of catalyzing the precipitation of Mn oxides may be useful for application in the removal and recovery of toxic metals from the environment. SG-1 spores are particularly well suited for this purpose for a variety of reasons (Tebo *et al.*, 1998). The oxidation of Mn(II) can occur over a wide range of environmental conditions and can accumulate on the surface of spores up to approximately 6 times their own weight under ideal conditions. In addition, the rates of Mn oxidation by SG-1 spores at neutral pH are over 4-5 orders of magnitude faster than abiotic Mn oxidation rates (Hastings and Emerson, 1986). Biological Mn(II) oxidation has actually been employed as an alternative to chemical oxidation in the removal of excess Mn from drinking water (Mouchet, 1992) as well as for the removal of toxic contaminants from mine drainage (Mathur *et al.*, 1988). A recent study also demonstrated that biogenic Mn oxides produced by *Leptothrix discophora* SS-1 have significantly greater surface area and Pb adsorption capacity than abiotically produced Mn oxide (Nelson *et al.*, 1999). This extremely high trace metal adsorption capacity of biologically produced Mn oxides provides yet another

advantage to employing Mn(II)-oxidizing bacteria for metal removal processes. Finally, the toxic metals adsorbed on the Mn oxides could be released by dissolving the oxides (e.g. with reducing agents) and the metals could be recovered and the spores recycled.

Conclusions and Future Directions

Bacteria play a central role in the biogeochemical cycling of metals in the environment, yet the molecular and biochemical mechanisms for most of these processes are not well understood. Clearly, the application of molecular biological approaches to the study of bacterial Mn(II) oxidation has transformed our understanding of this environmentally important process. However, it is important to recognize that this field is merely in its infancy and that major discoveries will surely be made in the very near future.

There are a number of important research avenues that should be pursued in future studies. Molecular analysis of other phylogenetically diverse Mn(II)-oxidizing bacteria using PCR primers and gene probes specific for known Mn(II) oxidation genes could reveal if multicopper oxidases are involved in all enzymatic Mn(II) oxidation systems. Functional gene probes for Mn(II) oxidation could then also be used to assess the distribution, abundance, and activities of Mn(II)-oxidizing organisms in the natural environment, even without the cultivation of organisms. Finally, more detailed biochemical and, especially, spectroscopic studies will be necessary to definitively elucidate the role of copper in the model Mn(II) oxidation systems. Overall, such studies should help further our understanding of the molecular basis (and function) of Mn oxidation, the factors which influence this environmentally important process, and how these organisms might be utilized to benefit society.

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