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Diversity and Molecular Mechanisms of Manganese(II)-Oxidizing Bacteria

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Diversity and Molecular Mechanisms of Manganese(II)-Oxidizing Bacteria

A dissertation in partial satisfaction of the

Requirements for the degree Doctor of Philosophy in

Marine Biology

by

Christopher A. Francis

Committee in charge:

Bradley M. Tebo
Douglas H. Bartlett
James A. Hoch
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2000

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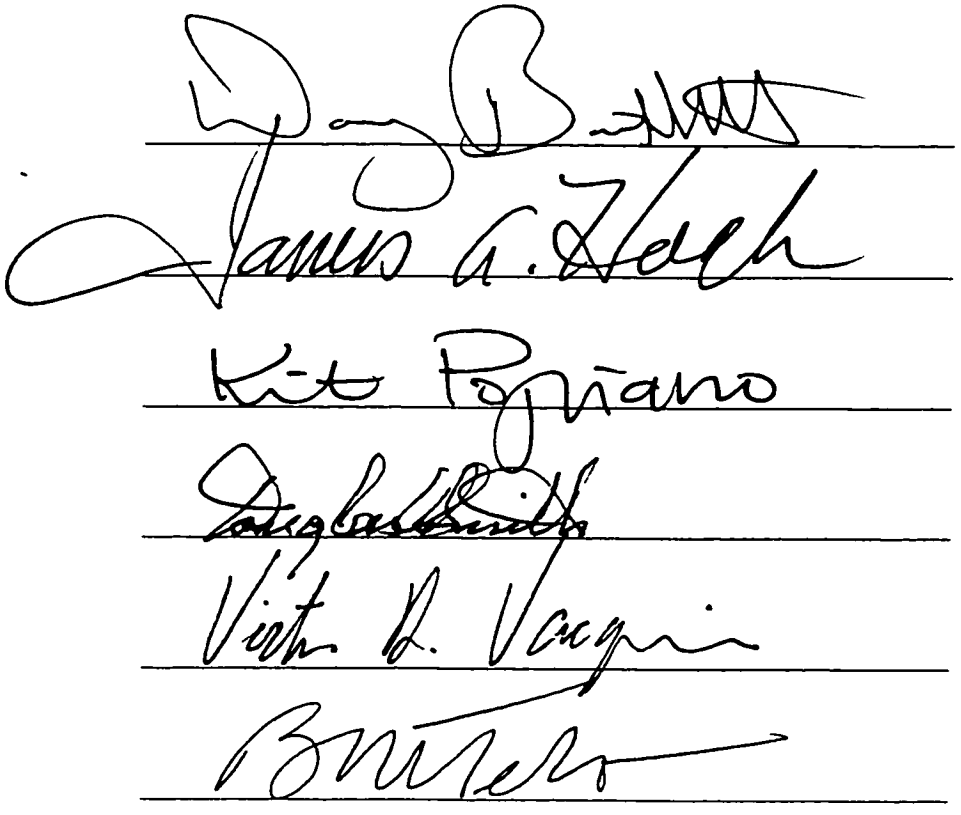

Chair

University of California, San Diego

2000

This work is dedicated to my family:

My parents Chick and Jean, my wife Alisa, and my precious daughter Natalie.

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instrumental in localizing the Mn(II)-oxidizing activity to the outermost layer of SG-1 spores and also isolated a number of the Mn(II)-oxidizing spore-formers described in this study. Eric Allen performed the fatty acid analysis of the outermost layer and spore coat fractions of SG-1 spores. I am indebted to Willie Swanson and Joseph Schulz for teaching me some of the finer points of working with proteins and antibodies. A number of undergraduates have also worked with me over the years: Dat Do, Tamarind Hamwood, Michael Lu, Azmi Nasir, and Edgie Co. I would especially like to thank Edgie Co for working long hours, running countless SDS-PAGE gels, and making a significant contribution to the characterization of the α-proteobacterium, SD-21.

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The text of Chapter two, in full, is a reprint of the material as it appears in the Journal of Microbiology and Biotechnology 1: 71-78. I was the primary author of this article and my advisor, Bradley Tebo, was the co-author.

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ABSTRACT OF THE DISSERTATION

Diversity and Molecular Mechanisms of
Manganese(II)-Oxidizing Bacteria

by

Christopher A. Francis
Doctor of Philosophy in Marine Biology
University of California, San Diego, 2000
Bradley M. Tebo, Chair

The marine *Bacillus* sp. strain SG-1 produces dormant spores that catalyze the oxidation of soluble Mn(II) to insoluble Mn(IV) oxides. The Mn(II)-oxidizing activity was localized to the ridged outermost spore layer, which could be physically removed and retain activity. MnxG, the product of the most downstream gene in the previously identified *mnx* gene cluster, is a multicopper oxidase believed to be the Mn(II) oxidase. MnxG was immunologically localized to the outermost layer of wild-type spores but was absent in non-oxidizing mutants within the *mnx* cluster, suggesting that MnxG may be the only protein directly involved in Mn(II) oxidation.

To explore the phylogenetic diversity of marine *Bacillus* strains capable of producing Mn(II)-oxidizing spores, 16S rRNA and *mnxG* sequences were PCR-

amplified from 14 Mn(II)-oxidizing spore-formers isolated from coastal marine sediments. Phylogenetic trees based on both genes revealed extensive diversity of Mn(II)-oxidizers within the genus *Bacillus*, including several distinct clusters and lineages. SDS-PAGE analysis of spore surface proteins revealed the presence of active Mn(II)-oxidizing bands which were inhibited by *o*-phenanthroline and azide, consistent with the involvement of a multicopper oxidase.

In *Pseudomonas putida* GB-1, a multicopper oxidase gene, *cumA*, is believed to encode an essential component of the outer membrane Mn(II)-oxidizing complex. Using degenerate PCR primers, homologous *cumA* sequences were amplified from a variety of Mn(II)-oxidizing and non-oxidizing *Pseudomonas* strains. Phylogenetic trees based on CumA and 16S rRNA sequences had similar topologies and revealed that the *cumA* gene and the capacity to oxidize Mn(II) occur in diverse *Pseudomonas* strains. However, *cumA* may have an alternative function or be functionally inactive in non-oxidizing strains.

A yellow-pigmented Mn(II)-oxidizing α-proteobacterium, strain SD-21, was isolated from surface sediments of San Diego Bay, CA. During logarithmic growth phase, this organism produces Mn(II)-oxidizing proteins which are heat labile and inhibited by azide and o-phenanthroline, suggesting the possible involvement of a multicopper oxidase. This is the first report of Mn(II) oxidation within the alpha-4-

Proteobacteria as well as the first Mn(II)-oxidizing protein(s) identified in a marine Gram-negative bacterium.

Overall, these studies indicate that 1) Mn(II)-oxidizing bacteria are phylogenetically diverse and that 2) copper may play a universal role in Mn(II) oxidation.

CHAPTER I

Introduction

The oxidation of soluble Mn(II) to highly insoluble Mn(III,IV) oxide and oxyhydroxide precipitates profoundly influences the distributions of metals, organics, and microorganisms in the environment. The Mn oxides produced through this reaction are extremely strong oxidants, highly effective scavengers of heavy metals, and important alternative electron acceptors for anaerobic respiration. Although bacteria are believed to be the primary catalysts of Mn(II) oxidation in most environments (Nealson et al., 1988), insights into the underlying molecular and biochemical mechanisms for this process have only recently started to emerge. This dissertation is focused on exploring both the diversity and molecular mechanisms of Mn(II)-oxidizing bacteria.

Mn Chemistry

Manganese is the second most abundant transition metal in the Earth's crust, and is most often found in nature in oxidation states ranging from +2 to +4. In the environment, the soluble manganous ion, Mn^{2+}, can be oxidized to insoluble Mn(III), Mn(IV), or mixed valent minerals, such as MnOOH (manganite), MnO_2 (vernadite), and Mn_3O_4 (hausmannite). A large number of structurallly diverse Mn oxide minerals exist (Tebo and He, 1999). These Mn oxides are ubiquitous and extremely important solid phases in soils, sediments, and waters (Post, 1999), where they generally occur

as grains and grain coatings. The highly charged and reactive surfaces of these oxides can oxidize a wide array of organic and inorganic compounds (e.g., aromatic hydrocarbons, humic acids, Cr(III), Fe(II), etc.) as well as scavenge numerous heavy metals (e.g., Cu, Co, Cd, Zn, Ni, Pb) and radionuclides out of solution (Hem, 1978; Murray, 1975). Thus, when Mn oxides are reduced (i.e., dissolved), many adsorbed metals may be released into the environment. Under aerobic conditions, the oxidation of Mn(II) is thermodynamically favorable above pH 6, but the kinetics of this reaction are extremely slow in the absence of a suitable catalyst (Stumm and Morgan, 1996). Mn(II) oxidation is autocatalytic, with the Mn oxide products adsorbing Mn(II) and catalyzing its further oxidation. In contrast to Fe(II), which undergoes rapid oxidation in the presence of oxygen to insoluble Fe(III) at circumneutral pH, Mn(II) is quite stable in the environment below pH 8.5.

Mn Biochemistry

Manganese is an essential element in all biological systems, functioning in over 20 different enzymes and proteins, including: ribonucleotide reductase, arginase, catalase, peroxidase, superoxide dismutase (SOD), and Photosystem II (PSII) (Crowley et al., 2000). Perhaps the most important biological roles of manganese are in the evolution of O_2 (PSII) and disposal of superoxide radicals (SOD). In fact,

photosynthetic water oxidation, which is the source for nearly all oxygen in the atmosphere and, thus, the indirect source for most biomass on Earth, is dependent on a multinuclear cluster of Mn ions within the catalytic site of PSII (Debus, 2000). Like many trace metals, Mn is generally only required in trace amounts for survival but can be toxic in higher concentrations. However, certain lactic acid bacteria are unusual in their ability to accumulate extremely high concentrations of manganese, which acts as an effective scavenger of superoxide. In particular, *Lactobacillus plantarum* accumulates 30-35 mM concentrations of manganese (Archibald and Fridovich, 1981a), thereby bypassing the requirement for a superoxide dismutase (Archibald and Fridovich, 1981b; 1982).

Bacterial Mn(II) Oxidation

One of many equations for the two-electron oxidation of Mn(II) to Mn(IV) is shown below:

$$Mn^{2+} + 0.5O_2 + H_2O \rightarrow MnO_2 + 2H^+ \qquad (\Delta G^{\circ\prime} = -70.9 \text{ kJ/mol})$$

When compared to the free energy required for the formation of a single ATP (-30 kJ/mol), it is clear that energy could potentially be gained from this reaction. However, the amount of energy actually available from this reaction depends on a number of factors, including: Mn(II) concentration, O_2 concentraion, pH, ionic

strength, and the type of mineral(s) formed. Since Mn concentrations in natural water environments can range from subnanomolar in the open ocean to millimolar in some sediment pore waters and freshwater streams (Morgan, 2000), it is more useful and informative to consider the free energy for Mn(II) oxidation at metal concentrations within this range. For example, under typical conditions found in the water column of the ocean ([Mn(II)]=1 nM, P_{O_2} = 0.2 atm, and pH 7.8), the ΔG° for Mn(II) oxidation would be considerably less (-25 kJ/mol) than the value calculated under standard conditions.

Although it is apparent from the thermodynamics that energy could theoretically be gained from Mn(II) oxidation, there have been no conclusive reports of organisms coupling this reaction to lithoautotrophic growth. Instead, all Mn(II)-oxidizers isolated to date appear to be heterotrophic organisms which apparently oxidize Mn(II) for non-energetic reasons. The Mn oxides produced by Mn(II)-oxidizing bacteria may serve a number of possible biological functions, including: protection against ultraviolet light, toxic metals, viral attack, predation, etc.; oxidants of refractory organic matter into utilizable substrates; scavengers of trace metals required for growth; and storage of an electron acceptor for anaerobic respiration (Tebo et al., 1997).

Due to the slow rates of abiotic Mn(II) oxidation under most environmental conditions, bacteria are believed to be the primary catalysts for this process in nature (Nealson et al., 1988; Tebo et al., 1997). A direct link between bacteria and the oxidative precipitation of manganese has been recognized for many years. In fact, two of the founding "fathers" of modern microbiology, Winogradsky and Beijerinck, studied the phenomenon of bacterial Mn(II) oxidation (Beijerinck, 1913; Winogradsky, 1888), yet this process is still not well understood today. Over the years, a wide variety of Mn(II)-oxidizing bacteria have been found in many different environments, including marine and freshwaters, wetlands, soils, sediments, hydrothermal vents, deep-sea Mn nodules, marine snow, desert varnish, and water pipes (Cowen and Silver, 1984; Douka, 1980; Ehrlich, 1968; Ghiorse, 1984; Gregory and Staley, 1982; Juniper and Tebo, 1995; Jung and Schweisfurth, 1979; Nealson, 1978; Schutt and Ottow, 1978; Tebo et al., 1997). These organisms are particularly prevalent at oxic-anoxic interfaces, such as those found in many fjords and marine basins (e.g., Black Sea; Saanich Inlet, B.C.), in which high amounts of reduced Mn(II) diffuses up from the anoxic zone and is readily oxidized by bacteria in the presence of oxygen (Tebo, 1991; Tebo et al., 1985).

In addition to being environmentally ubiquitous, bacteria capable of Mn(II) oxidation are phylogenetically diverse, with all known organisms falling within the

low-G+C Gram-positive, Actinobacteria, or Proteobacterial lineages of the domain Bacteria. Some of the genera for which Mn(II)-oxidizing strains have been reported include *Bacillus*, *Arthrobacter*, *Streptomyces*, *Pseudomonas*, *Vibrio*, *Leptothrix*, *Aeromonas*, *Hyphomicrobium*, *Pedomicrobium*, *Caulobacter* (Ghiorse, 1984; Tebo and He, 1999) Despite the extensive diversity of bacteria capable of oxidizing Mn(II), the underlying mechanisms of bacterial catalysis have only been thoroughly studied in a few model organisms: the marine *Bacillus* sp. strain SG-1, *Leptothrix discophora* SS-1, and *Pseudomonas putida* strains GB-1 and MnB1.

Model Organisms

***Bacillus* sp. strain SG-1.** The marine *Bacillus* sp. strain SG-1 was originally isolated from a shallow marine sediment off Scripps Pier, based on its ability to form brownish-black, Mn oxide-encrusted colonies on Mn(II)-containing agar plates (Nealson and Ford, 1980). This Gram-positive organism is unique in that it produces metabolically dormant spores that directly catalyze the oxidation of Mn(II) on the spore surface. In fact, SG-1 spores can increase the rate of Mn(II) oxidation by 4-5 orders of magnitude relative to abiotic rates (Hastings and Emerson, 1986). Early studies demonstrated that the spore-associated activity was heat-labile and poisoned by various enzymatic inhibitors (Rosson and Nealson, 1982), suggesting that Mn(II)

oxidation was protein-catalyzed. Transmission electron microscopic analysis of SG-1 spores revealed that Mn oxides accumulated on the ridged outermost layer, either a spore coat or exosporium (Tebo, 1983). In addition, de Vrind et al. (1986) demonstrated that spore coat preparations, processed to retain all the outer layers and remove spore contents, retained full Mn(II)-oxidizing activity. In an effort to isolate the Mn(II)-oxidizing protein(s) from spores, previous studies employed a combination of harsh chemical conditions (e.g., pH 9.8; 8M urea; 50 mM dithiothreitol; 1% SDS) to solubilize the spore surface proteins, which were subsequently analyzed for activity in SDS-PAGE gels incubated in Mn(II). Although these methods occasionally led to the recovery of high-molecular-weight Mn(II)-oxidizing bands in gels (Tebo et al., 1988), it was difficult to reproducibly obtain sufficient quantities of active protein for biochemical analysis.

To circumvent the difficulties associated with the biochemical approach, van Waasbergen et al. (1993) developed a genetic system in SG-1 and used transposon mutagenesis to generate mutants that produced spores incapable of Mn(II) oxidation. Analysis of these mutants resulted in the identification of a cluster of genes, the *mnx* genes, apparently essential for Mn(II) oxidation (van Waasbergen et al., 1996). Sequence analysis revealed that the most downstream gene in this cluster, *mnxG*, encoded a 138 kDa protein predicted to be the Mn(II) oxidase. MnxG is related to

multicopper oxidases, a diverse group of proteins that utilize multiple copper ions as cofactors in the oxidation of a wide variety of substrates, including organics and in several cases Fe(II) (Solomon et al., 1996). Well-known multicopper oxidases include ascorbate oxidase, laccase, and ceruloplasmin, all of which are primarily found in eukaryotic organisms. Biochemical evidence for the involvement of a multicopper oxidase in SG-1 spores comes from the fact that the Mn(II)-oxidizing activity of these spores is enhanced by small concentrations of copper (~1 μM) (van Waasbergen et al., 1996) and inhibited by azide (Rosson and Nealson, 1982), a potent inhibitor of multicopper oxidases.

***Leptothrix discophora* SS-1.** *Leptothrix discophora* oxidizes Mn(II) via a protein associated with the outermost sheath layer. This layer, a complex heteropolymer held together primarily by disulfide bonds (Emerson and Ghiorse, 1993), has an excess of available COOH and SH groups which provide potential metal-binding ligands. In the sheathless variant of *L. discophora*, strain SS-1, the Mn(II)-oxidizing protein is excreted into the culture medium, which leads to the eventual formation of Mn oxide particles free in the medium (Adams and Ghiorse, 1986). Mn(II)-oxidizing proteins have been isolated from both sheathed (SP-6) and non-sheathed (SS-1) strains of *L. discophora*. In both cases, a ~110-kDa Mn(II)-oxidizing protein was consistently recovered in SDS-PAGE gels incubated in Mn(II)

(Adams and Ghiorse, 1987; Boogerd and de Vrind, 1987; Emerson and Ghiorse, 1992). The activity of this enzyme was heat-labile and inhibited by various poisons (e.g., azide, potassium cyanide), but quite stable in the presence of SDS.

As in the *Bacillus* sp. strain SG-1, difficulties were encountered in purifying large quantities of active protein from these organisms for biochemical analysis. However, Corstjens et al. (1997) was able to obtain sufficient quantities of protein, via electrolution of protein bands from SDS-PAGE gels, to develop specific antisera to the Mn(II)-oxidizing protein of strain SS-1. By screening an expression library with this antibody, the putative gene for the Mn(II)-oxidizing factor was cloned and sequenced (Corstjens et al., 1997). This gene, designated *mofA*, encodes a174-kDa protein that is related to multicopper oxidases. The discrepancy in size between the predicted protein and the active protein in gels may be due to proteolysis or partial degradation of the full-length protein, while retaining activity (Corstjens et al., 1997). Additional evidence for proteolysis comes from the fact that active Mn(II)-oxidizing proteins as small as 85 kDa have occasionally been observed in SDS-PAGE gels as well (Boogerd and de Vrind, 1987). Although both MofA and MnxG are related to multicopper oxidases, there is little to no similarity between these sequences outside of the conserved copper-binding regions. Recently, it has been demonstrated that the Mn(II)-oxidizing activity of *L. discophora* is enhanced by the presence of copper in

the culture medium (Brouwers et al., 2000), supporting involvement of a multicopper oxidase in this organism.

***Pseudomonas putida* GB-1 and MnB1.** *P. putida* GB-1 and MnB1 are closely related strains that catalyze the oxidation of Mn(II) during the onset of stationary phase and/or during starvation (De Palma, 1993; Jung and Schweisfurth, 1979). Mn(II)-oxidizing factors have recently been partially purified from GB-1 which have approximate sizes of 250-kDa and 180-kDa in native polyacrylamide gradient gels (Okazaki et al., 1997). In contrast to the *Bacillus* sp. strain SG-1 and *L. discophora*, these proteins are more sensitive to SDS, possibly because they are mulit-protein complexes which may be disrupted by detergents. The activity of these factors was inhibited by azide as well as low concentrations (10 μM) of the copper-chelator, *o*-phenanthroline, consistent with the involvement of a metalloenzyme (e.g., mulitcopper oxidase) (Okazaki et al., 1997).

In an effort to identify genes involved in Mn(II) oxidation, transposon mutagenesis was employed to generate non-oxidizing mutants in both *P. putida* GB-1 and MnB1 (Caspi et al., 1998; de Vrind et al., 1998). A number of genes were identified which were apparently required for Mn(II) oxidation, although some of them are believed to be only indirectly involved in this process, including TCA cycle genes, protein secretion pathway genes, and tryptophan biosynthesis genes. In both

organisms, genes involved in the biogenesis and maturation of *c*-type cytochromes (the *ccm* genes) were identified, suggesting that cytochromes may be important components of the Mn(II)-oxidizing complexes. However, a gene related to multicopper oxidases, designated *cumA*, was also found to be essential for Mn(II) oxidation in GB-1 (Brouwers et al., 1999; de Vrind et al., 1998). The predicted 50-kDa product of the *cumA* gene is believed to be a Cu-dependent oxidase which is the key component of the Mn(II)-oxidizing complex. In addition, the presence of copper in the growth medium was found to enhance the Mn(II)-oxidizing activity of GB-1 (Brouwers et al., 1999). A direct role for both cytochromes and CumA in Mn(II) oxidation was supported by the fact that Mn(II)-oxidizing complexes were completely absent from these mutants in protein gels (de Vrind et al., 1998).

Multicopper Oxidases and Mn(II) Oxidation

When comparing the mechanisms of Mn(II) oxidation of the phylogenetically distinct model organisms described above, it is quite evident that they are all linked by the involvement of multicopper oxidases in catalyzing this reaction. In addition, azide, a potent metalloprotein inhibitor which bridges the type 2 and type 3 copper atoms of multicopper oxidases, is inhibitory to all three systems, while copper has been shown to actually enhance the Mn(II)-oxidizing activity of these systems.

Recently, it has also been demonstrated that Mn(II) oxidation by the α-proteobacterium, *Pedomicrobium* ACM3067 appears to be catalyzed by a copper-dependent enzyme (Larsen et al., 1999). Thus, it possible that copper may play a universal role in bacterial Mn(II) oxidation. However, it has yet to be determined how widespread both multicopper oxidases and the ability to oxidize Mn(II) are within the environmentally-important genera, *Bacillus* and *Pseudomonas*, as well as the α-Proteobacteria.

Organization of the Dissertation

The motivation of this research was to explore the molecular and biochemical mechanisms of bacterial Mn(II) oxidation through studies of several phylogenetically distinct groups of bacteria: the Gram-positive, spore-forming genus, *Bacillus*; the Gram-negative, γ-proteobacterial genus, *Pseudomonas*; and a marine α-proteobacterium. This dissertation is organized into six subsequent chapters that are summarized below:

Chapter 2 reviews the current molecular and biochemical mechanisms of Mn(II) oxidation by spores of the marine *Bacillus* sp. strain SG-1. Comparisons are also made with multicopper oxidases as well as the model Mn(II)-oxidizing organisms, *Leptothrix discophora* SS-1 and *Pseudomonas putida* strains GB-1 and

MnB1. Finally, the potential biotechnological applications of Mn(II)-oxidizing bacteria are discussed.

Chapter 3 describes the localization of Mn(II)-oxidizing activity and the multicopper oxidase, MnxG, to the outermost layer of spores of the marine *Bacillus* sp. strain SG-1. Mn(II) oxidation is the first oxidase activity, and MnxG one of the first gene products, ever shown to be associated with such a layer. The French press was found to be an effective method for physically removing the Mn(II)-oxidizing outer layer from spores while retaining activity.

Chapter 4 explores the phylogenetic diversity of marine *Bacillus* strains capable of producing Mn(II)-oxidizing spores . Phylogenetic analysis of 16S rRNA genes as well as MnxG multicopper oxidase sequences obtained from a number of Mn(II)-oxidizing spore-formers revealed extensive diversity within the genus *Bacillus*. Active Mn(II)-oxidizing proteins were recovered in SDS-PAGE gels from all of the isolates, the activity of which was inhibited by both *o*-phenanthroline and azide, consistent with the involvement of multicopper oxidases in these phylogenetically diverse *Bacillus* strains.

Chapter 5 addresses how widespread the ability to oxidize Mn(II) is within the genus *Pseudomonas*. Phylogenetic analysis was performed on a variety of Mn(II)-oxidizing and non-oxidizing *Pseudomonas* strains, based on 16S rRNA sequences as

well as the functional gene, *cumA*, which encodes the multicopper oxidase essential for Mn(II) oxidation in *P.putida* GB-1. Both the *cumA* gene and the capacity to oxidize Mn(II) occur in diverse *Pseudomonas* strains, but the *cumA* gene does not appear to be restricted to Mn(II)-oxidizing strains..

Chapter 6 reports the characterization of a yellow-pigmented, Mn(II)-oxidizing α-proteobacterium isolated from coastal marine sediments. This organism produces a stable Mn(II)-oxidizing protein complex which is heat labile and inhibited by both azide and o-phenanthroline, suggesting the involvement of a multicopper oxidase. This is the first report of Mn(II) oxidation within the alpha-4-subgroup of the *Proteobacteria* and the first Mn(II) oxidase identified in a marine Gram-negative bacterium.

Chapter 7 synthesizes the results from the previous chapters and draws conclusions regarding these data. A number of future research directions are also discussed.

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CHAPTER II

Marine *Bacillus* Spores as Catalysts for Oxidative

Precipitation and Sorption of Metals

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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 µm.

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 Nealson, 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 Nealson, 1982). The oxidizing activity of the spores is heat labile and is poisoned by the metalloprotein inhibitors azide, cyanide, and mercuric chloride (Rosson and Nealson, 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 Tn*917*, 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.

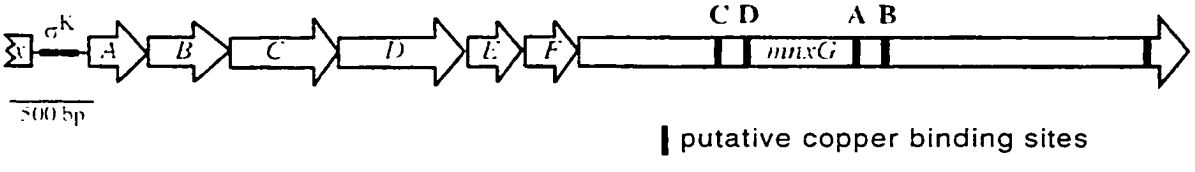

Figure 2. The *mnx* Gene Cluster
The organization of the *mnx* gene cluster of the marine *Bacillus* sp. strain SG-1 based on DNA sequence analysis. *mnxG* encodes the putative Mn(II)-oxidizing protein which shares significant similarity with multicopper oxidases, particularly in the regions of copper 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 ß-galactosidase activity from a Tn*917-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:

$$\text{Substrate} \xrightarrow{e^-} \text{Type 1} \xrightarrow{e^-} \{\text{Type 2} + (\text{Type 3})_2\} \xrightarrow{e^-} O_2$$

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

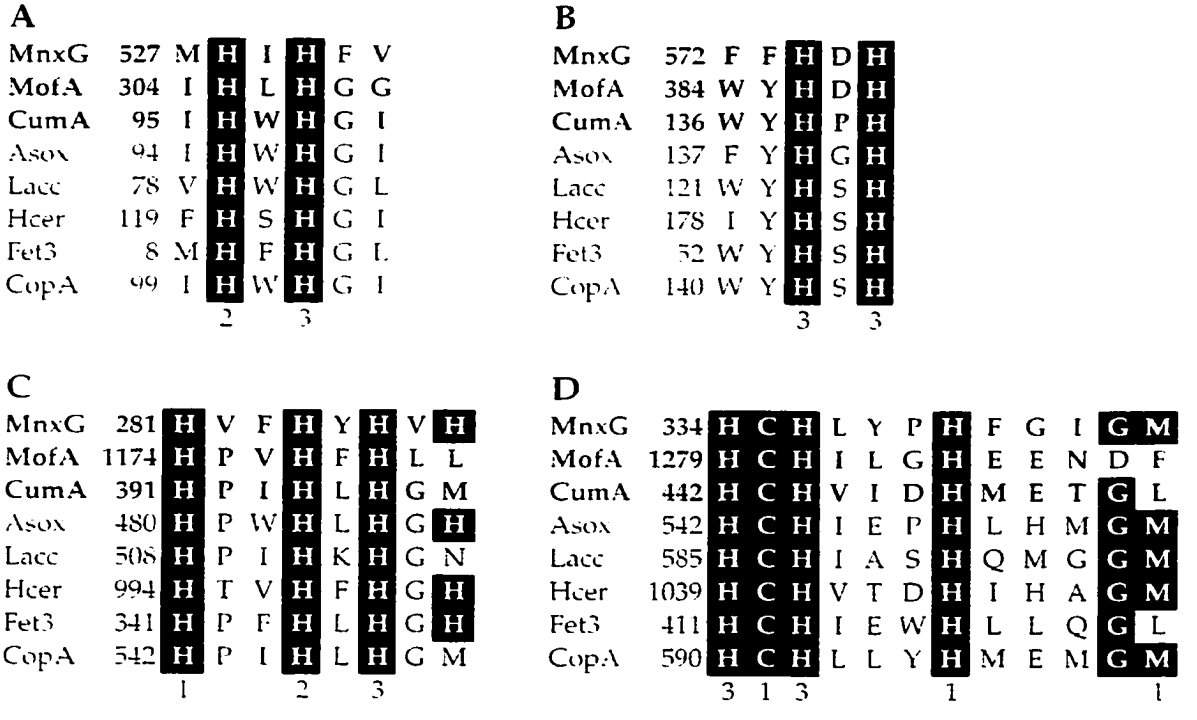

Figure 3. Copper-binding Sites in Multicopper Oxidases
Amino acid alignment of the copper-binding sites in MnxG, MofA, CumA, and other multicopper oxidases. The letters A-D correspond to the copper binding sites shown in Figure 2. Abbreviations: Asox, ascorbate oxidase (cucumber and squash); Lacc, laccase (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-innibition 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 m^2g^{-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

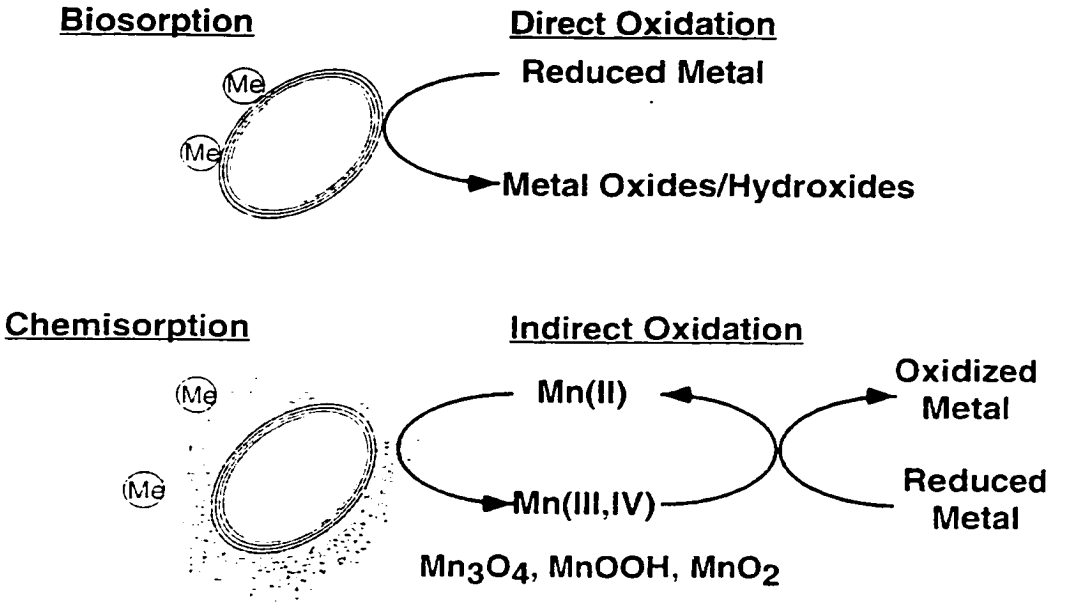

Figure 4. Schematic Representation of the Mechanisms by which SG-1 Spores can Either Adsorb or Oxidize Various Metals
Top: The spores can passively adsorb certain metals (where **Me** = Cu, Cd, Zn, Ni) on the charged spore surface (biosorption). The enzymatic activity of the outermost spore layer can 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 2.08×10^6 L·mol^{-1} and 10.77 µmol·m^{-2} respectively. The K value is simply the inverse of the substrate binding constant (K_s = 0.48 µM) with which most

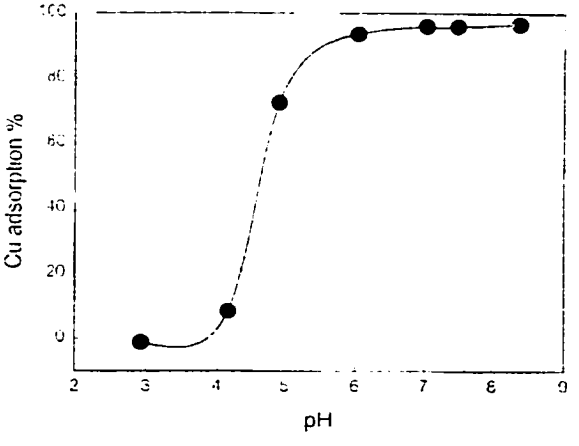

Figure 5. Cu(II) Adsorption by SG-1 Spores
Cu(II) adsorption by SG-1 spores as a function of pH [10^8 spores ml^{-1}, 0.01 M $NaNO_3$, 2 µM Cu(II)]. The adsorption value for 100% adsorption is 1.5 µmol m^{-2}. Reproduced with permission from He and Tebo (1998).

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 ($K_M = 3.3 \times 10^{-8}$ M; $V_{max} = 1.7 \times 10^{-15}$ M^{-1}·h^{-1}) and a low-

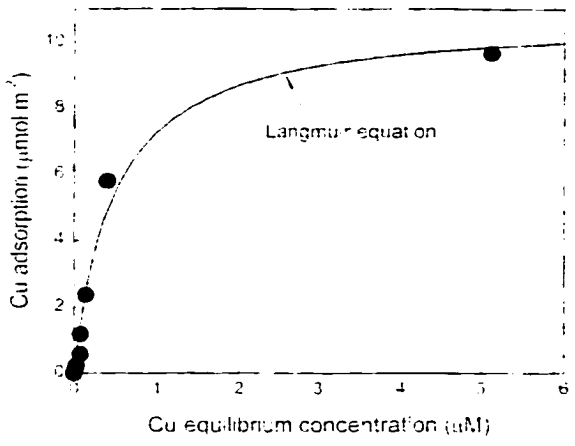

Figure 6. Cu(II) Adsorption Isotherm
Cu(II) adsorption isotherm obtained with SG-1 spores (10^{11} spores ml^{-1}, 0.01 M $NaNO_3$, pH 7.0). The curve was obtained by fitting the data to the Langmuir equation. The results demonstrate that the spores have both a high affinity (K_L = 0.48 μM) and a high adsorption capacity for Cu (10.77 μmol m^{-2} of spore surface or 0.83 mmol g^{-1} dried spores). Reproduced with permission from He and Tebo (1998).

affinity-high-rate system (K_M = 5.2 x 10^{-6} M; V_{max} = 8.9 x 10^{-15} M^{-1} 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.

Acknowledgements

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CHAPTER III

Localization of Mn(II)-Oxidizing Activity and the Multicopper Oxidase, MnxG, to the Outermost Spore Layer of the Marine *Bacillus* sp. strain SG-1

ABSTRACT

Dormant spores of the marine *Bacillus* sp. strain SG-1 catalyze the oxidation of manganese(II), thereby becoming encrusted with insoluble Mn(III,IV) oxides. In this study, it was found that the Mn(II)-oxidizing activity could be removed from SG-1 spores using a French press and recovered in the supernatant following centrifugation of the spores. Transmission electron microscopy of thin sections of SG-1 spores revealed that the ridged outermost layer was removed by passage through the French Press, leaving the remainder of the spore intact. Comparative chemical analysis of this layer with the underlying spore coats suggested that this layer is chemically distinct from the spore coat, possibly representing an exosporium. Previous genetic analysis of strain SG-1 identified a cluster of genes involved in Mn(II) oxidation, the *mnx* genes. The product of the most downstream gene in this cluster, MnxG, appears to be a multicopper oxidase and is essential for Mn(II) oxidation. In this study, MnxG was overexpressed in *Escherichia coli* and used to generate polyclonal antibodies. Western blot analysis demonstrated that MnxG is localized to the outermost layer of wild-type spores but is absent in the non-oxidizing spores of transposon mutants within the *mnx* gene cluster. To my knowledge, Mn(II) oxidation is the first oxidase

activity, and MnxG one of the first gene products, ever shown to be associated with an outermost spore layer.

INTRODUCTION

Mature spores of the marine *Bacillus* sp. strain SG-1 oxidize soluble manganese [Mn(II)], thereby becoming encrusted with highly insoluble Mn(IV) oxide precipitates. Since first isolated from a shallow marine sediment off Scripps pier over 20 years ago (Nealson and Ford, 1980), SG-1 has been studied as a model Mn(II)-oxidizing organism (Francis and Tebo, 1999; Tebo et al., 1997). Transmission electron microscopy demonstrated that the Mn oxides precipitate on the ridged outermost spore layer (Tebo, 1983), and spore coat preparations, processed to retain all the outer layers and remove spore contents, were shown to retain full oxidizing activity (de Vrind et al., 1986). The Mn(II)-oxidizing activity was shown to be heat labile and poisoned by metalloprotein inhibitors (Rosson and Nealson, 1982), suggesting that a metalloenzyme associated with the spore surface, either the spore coat or an exosporium, was responsible for catalyzing this reaction. However, the inability to isolate large quantities of active protein from spores hampered detailed biochemical analysis and localization of the Mn(II)-oxidizing factor(s).

In an effort to identify genes involved in Mn(II) oxidation, methods for plasmid transformation and transposon mutagenesis were developed in SG-1 (van Waasbergen et al., 1993). By generating mutants which produced spores incapable of oxidizing Mn(II), a cluster of seven genes involved in Mn(II) oxidation, the *mnx* genes, were identified (van Waasbergen et al., 1996). Sequence analysis demonstrated that one of the encoded proteins, MnxG, was the probable candidate for the Mn(II)-oxidizing protein. MnxG shows similarity to multicopper oxidases, a diverse group of proteins which utilize multiple copper ions as cofactors in the oxidation of a variety of substrates (Ryden and Hunt, 1993; Solomon et al., 1996).

Recently, genes involved in Mn(II) oxidation have also been identified in two other bacteria, *Leptothrix discophora* SS-1 and *Pseudomonas putida* GB-1, and in both cases, enzymes related to multicopper oxidases appear to be involved (Corstjens et al., 1997; Brouwers et al., 1999). The involvement of multicopper oxidase-like enzymes in three phylogenetically distinct Mn(II)-oxidizing organisms suggests that copper may play a universal role in enzymatic Mn(II) oxidation. The objective of this study was to definitively localize the Mn(II)-oxidizing activity of SG-1 spores and to further elucidate the role of the multicopper oxidase, MnxG, in Mn(II) oxidation.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. The bacterial strains used in this study were the *Bacillus* sp. strain SG-1 and various *Tn*917 manganese oxidation mutants generated by van Waasbergen et al. (1993). The sporulation medium for SG-1 is a modified K medium (Rosson and Nealson, 1982) containing 2.0 mg of peptone (Difco) per ml and 0.5 mg of yeast extract (Difco) per ml in sterile seawater with both 20 mM HEPES (pH 7.6) and 100 μM $MnCl_2$ added after autoclaving.

Isolation of Mn(II)-oxidizing layer. Spores were purified by the method of Rosson and Nealson (1982). The outermost layer was isolated by passing purified spores through a French pressure cell six times at 20,000 psi. Stripped spores were then removed by centrifugation at 14,000 x g, and the supernatant, containing the activity, was recovered. Supernatant was assayed for Mn(II)-oxidizing activity by incubation in 10 mM HEPES (pH 7.6) containing 100μM $MnCl_2$ and observing the formation of brown Mn oxides.

Electron microscopy of spores. Purified spores (untreated and French press-treated) were incubated in 10 mM HEPES-buffered distilled water with 100 μM $MnCl_2$ for 3 h at room temperature and fixed in glutaraldehyde/cacodylate buffer at

4°C overnight. Fixed spores were then washed twice with cacodylate buffer, postfixed in 1% OsO_4, embedded in agar, dehydrated through a graded ethanol series (30%, 50%, 60%, 70%, 80%, 90%, 3x95%, 3x100%), and embedded in Spurr's resin. Blocks were sectioned, stained with 2% uranyl acetate and Reynold's lead citrate, and viewed with a JEOL1200-EXII Transmission Electron Microscope at 60 kV accelerating voltage.

Chemical composition analysis. For chemical compositional analysis, the outermost layer was removed from 4 L (culture volume) of SG-1 spores as described above, and lyophilized. Spore coats were obtained from the French press-stripped spores essentially according to the method of de Vrind et al. (1986). Briefly, spores were sonicated with glass beads to break open the spores, lysozyme-treated to digest the cortex, and washed extensively using the series of buffers used in the spore purification protocol (Rosson and Nealson, 1982). The spore coat fraction was analyzed microscopically to ensure that no spores were present and then lyophilized. Amino acid composition was determined by the Stanford Protein and Nucleic acid (PAN) Facililty. Samples were acid hydrolyzed with 6N HCl under vacuum for 24 h at 110°C, followed by performic acid oxidation and analysis using a Beckman 6300 Amino Acid Analyzer. Neutral and amino sugar analysis was performed by the UC

San Diego Glycotechnology Core Facility. Samples were hydrolyzed in 2M trifluoroacetic acid at 120°C for 3 h and analyzed by HPLC. The response was normalized to standards of glucose, galactosamine, glucosamine, xylose, rhamnose, and mannose subjected to the same hydrolysis treatment. Lipids were extracted by the standard method of Bligh and Dyer (1959) in a choroform/methanol/water system. Total lipid content was determined by charring in H_2SO_4 at 200°C followed by spectrophotometric quantification against tripalmitin standards (Marsh and Weinstein, 1966).

Prior to fatty acid methyl ester (FAME) derivatization, samples (outer layer, spore coat, and vegetative cell pellets) were frozen at -80°C overnight and lyophilized. FAME analysis of this material was performed as described previously (Allen et al., 1999). FAMEs were prepared by reacting 2.5 to 10 mg (dry weight) of lyophilized sample with 5% H_2SO_4 in anhydrous methanol at 90°C for 90 minutes in 1.5 ml sample vials with Teflon-lined caps. FAMEs were extracted twice with hexane and nonesterified fatty acids were saponified with 10% NaCl. Hexane layers were removed and evaporated under a gentle steam of N_2 prior to analysis. Analysis of the FAME preparations was performed on a Hewlett-Packard model 5890 gas chromatograph equipped with an Econo-Cap EC-Wax capillary column (30m by 0.25 mm ID by 0.25 micron) connected to an HP model 5988A mass spectrometer.

Compounds were identified by comparison of their retention times with those of known standards and sample mass spectra data were compared to the mass spectra of known standards. Fatty acids are denoted as number of carbon atoms:number double bonds. Branched chain fatty acids were not resolved to iso- and anteiso- species and have been combined as branched chain fatty acids only.

SDS-PAGE analysis. Supernatant containing the outermost layer was mixed with 2X Laemmli buffer (Laemmli, 1970) and proteins were separated by SDS-PAGE in standard 6% gels or gradient gels (2.5 to 15%), followed by staining with Coomassie blue. To assay for in-gel Mn(II)-oxidation activity, gels were first incubated in 0.5% Triton-X-100/10% glycerol for 30 min to remove SDS, and then incubated in 10 mM HEPES buffer (pH 7.6) containing 100 μM $MnCl_2$. Mn(II) oxidation was visualized by the formation of brown Mn oxide bands in gels after several hours of incubation.

Overexpression of MnxG and generation of antibodies. The *mnxG* gene was amplified with PCR primers containing engineered *Kpn1* sites, restriction digested, and cloned into the expression vector pTRXFus (Invitrogen), creating an in-frame fusion with the *E. coli* thioredoxin gene. The fusion protein expressed from tryptophan-induced cells, which constituted most of the insoluble fraction of the *E. coli* cell lysates, was electrophoresed in 6% SDS-polyacrylamide gels, and bands of

the MnxG fusion protein were excised from KCl-stained gels and used for the production of polyclonal antisera in chickens (Cocalico Biologicals, Inc.). Antibodies were preadsorbed with an acetone powder of *E. coli* (pTRXFus) in which thioredoxin had been expressed, followed by affinity purification using expressed MnxG immobilized on nitrocellulose blots (Harlow and Lane, 1988).

Western blot analysis. Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose for Western blot analysis. Prestained molecular weight markers (Sigma) were used as standards. The primary antibody used for probing Western blots was purified anti-MnxG chicken polyclonal antiserum (1:1000), and the secondary antibody was a rabbit anti-chicken-IgY horseradish peroxidase conjugate (Jackson). Blots were developed using enhanced chemiluminescence (DuPont-NEN).

RESULTS AND DISCUSSION

Localization of Mn(II)-oxidizing activity. The Mn(II)-oxidizing activity of SG-1 spores was reduced by approximately 50% after several passages through a French press at 20,000 psi, centrifugation, and removal of the supernatant. The activity lost from the spores was recovered in the supernatant (Fig.1), indicating that

some component of the spores that was responsible for the activity was being removed or stripped off. French press-treatment has been recognized as an effective method for removing the exosporium, an outermost spore layer found in certain *Bacillus* and *Clostridium* species, without disrupting the integrity of the remaining spore layers (e.g., spore coat) (Charlton et al., 1999; Matz et al., 1970). Previous studies of SG-1 indicated that Mn(II) oxidation was catalyzed by a protein component on the spore surface (deVrind et al., 1986); however, no further localization of this activity to the spore coat or an exosporium was made. In this study, the removal of Mn(II)-oxidizing activity from SG-1 spores by mechanical shear suggested that an exosporium protein might be responsible for this activity.

The effect of French-press treatment on the ultrastructure of SG-1 spores was visualized by transmission electron microscopy (Fig.2). SG-1 spores are similar in appearance to *B. subtilis* spores but with an additional ridged layer outside the electron-dense spore coats. Incubation of the spores in a Mn(II) buffer prior to fixation resulted in the formation of Mn oxides, visible as black precipitates, on the spore surface (Fig.2a). The pronounced ridges, which are clearly associated with the Mn oxides in the untreated spores, were almost completely removed in the French-press treated spores (Fig.2b). However, some of the outermost layer was still present after French press-treatment, as evidenced by the small patches of Mn oxides still

associated with the spore surface. These patches may represent attachment sites or anchors for this outer layer, which are not easily removed by mechanical shear. It should also be pointed out that not all spores passed through the French press are completely stripped of the outermost layer. In fact, Matz et al. (1970) found that, after passage of *B. cereus* spores through a pressure cell at 32,000 psi, only 35% were totally stripped, 50% were partially stripped, and the remainder were left intact. The fact that the outermost layer is only partially removed by the French press explains why some activity is still associated with spores following this treatment.

The spore coat is a highly cross-linked proteinaceous structure that gives the spore resistance to chemical attack and mechanical disruption (Driks, 1999; Warth, 1978). The exosporium is an additional layer found only in certain *Bacillus* and *Clostridium* species and is defined as a loose-fitting, membranous layer, composed of protein, lipid, and carbohydrate (Matz et al., 1970; Tipper and Gauthier, 1972). Chemical analysis of the outermost layer of SG-1 spores demonstrated that it was composed of protein, carbohydrate, and lipid (TABLE 1). Although it is evident from TEM that the spore coat fraction is heavily contaminated with the outermost layer, these two layers had some distinguishing chemical properties. There were a number of minor differences in the relative amino acid compositions of the outermost layer and the spore coat as well as few more notable differences (TABLE 2). Based on

molar ratios, the outermost layer appeared to have higher amounts of leucine and isoleucine. In addition, there was a higher concentration of tyrosine in the spore coat, which is consistent with previous studies of *B. subtilis* indicating the presence of tyrosine-rich spore coat proteins (Driks, 1999), as well as dityrosine crosslinks in this layer (Pandey and Aronson, 1979). The primary monosaccharides present in both layers were very similar in type and abundance. The sugars found (in order of decreasing abundance) were glucosamine, galactosamine, glucose, mannose, galactose, and xylose. The absence of muramic acid in both layers suggests lack of contamination with cortical glycopeptide. The crude lipid content was also similar in both layers. The similar amounts of lipid and carbohydrate in both layers may be due in part to incomplete removal of the outermost layer by the French press. There is some uncertainty regarding what may constitute the remainder of the dry weight of the outermost layer. Previous studies of exosporia have indicated the presence of minor components such as ash, nucleic acids, teichoic acid, ammonia, and phosphorus (Matz et al., 1970). Evidence for the presence of phosphorus in the outermost layer of SG-1 spores comes from previous studies which predicted that phosphate and carboxylate groups were primarily responsible for the surface charge and copper adsorption capacity of the spore surface (He and Tebo, 1998). Fatty acid methyl ester (FAME) analysis revealed distinct differences in the fatty acid composition of the two layers

(TABLE 3). The spore coat had significantly greater relative amounts of branched-14:0, branched-16:0, and 16:0 fatty acids, whereas the outermost layer contained a much higher percentage of 15:0 and branched-16:1 fatty acids. The amino acid and fatty acid data suggest that the spore coat and outermost layer are chemically distinct layers.

The gross chemical composition of the *B. cereus* exosporium (Matz et al., 1970) is 52% protein, 20% polysaccharide, 18% lipid, and 4% ash. Compared to that layer, the outermost layer of SG-1 spores contains an almost identical amount of total protein (52.62%) as well as fairly comparable molar ratios of amino acids. These include essentially identical ratios for phenylalanine and leucine, both of which are clearly higher in concentration within the outermost layer than in the spore coat of SG-1 spores. Despite the similarities between the protein fractions of the SG-1 outermost layer and the *B. cereus* exosporium, considerably lower amounts of polysaccharide (4.85%) and lipid (6.3%) were detected in the SG-1 outer layer. Of the sugars found in the *B. cereus* exosporium (glucose, rhamnose, glucosamine, and ribose), only glucose and glucosamine were also present in the outer layer of SG-1, which also contained large amounts of galactosamine and smaller amounts of mannose, galactose, and xylose. Comparisons are made with *B. cereus* because this is the only organism for which the chemical composition of the exosporium has been determined. It is

possible that the differences in the outermost spore layers of SG-1 and *B. cereus* are due partly to the fact that these organisms are not closely related phylogenetically (see Chapter 4) and, thus, might be expected to have significant physiological differences, including exosporium composition.

Although no definite function has been attributed to exosporia, a number of possible functions have been proposed over the years. One of the most obvious potential functions of the exosporium is to provide an additional layer of defense to the perimeter of the spore. Du and Nickerson (1996) proposed that the insecticidal crystal (Cry) toxins of some *B. thuringiensis* species are protected from the environment by enclosure within the exosporium. Experimentally, this was supported by the fact that the exosporium had to be removed or permeabilized to achieve binding to either toxin-directed antibodies or to toxin receptor(s) from insect brush border membrane vesicles (BBMVs). However, in a study by Nicolas et al. (1994), no significant difference was found between the environmental stability of the *B. sphaericus* insecticidal protein when expressed inside or outside of the exosporium. Thus, it remains unclear to what extent the exosporium plays a protective role in nature. Another possible function stems from results of a study by Koshikawa et al. (1989) in which spores with exosporia were found to have greater hydrophobicity than spores lacking this layer. This increased hydrophobicity could potentially facilitate

attachment/adhesion to surfaces (Charlton et al., 1999). Overall, it seems somewhat unlikely that the exosporium has an indispensable function since it is not present in spores of all *Bacillus* species. However, the exosporium may have species-specific functions which somehow relate to the ecological niche(s) of a given organism.

Compared to the *Bacillus* spore coat, very little is known regarding the identities of the proteins localized within the exosporium. However, in a recent study, N-terminal sequences were obtained from several proteins associated with the exosporium of *B. cereus* ATCC 10876 (Charlton et al., 1999). Comparisons with the protein databases revealed several homologues, including: a zinc-metalloprotease (InA), already described in *B. thuringiensis*; a molecular chaperone (GroEL); and a homologue of RocA (1-pyroline-5-carboxylate dehydrogenase) from *B. subtilis*. The functions of these proteins, or how tightly they are associated with the exosporium of *B. cereus*, is currently unknown.

MnxG localization. In previous genetic studies of the marine *Bacillus* sp. strain SG-1, transposon mutants were generated which produced spores incapable of Mn(II) oxidation (van Waasbergen et al., 1993). Transmission electron microscopic analysis of these mutants revealed subtle changes in the outermost spore layer (e.g., more loosely attached, peeling/sloughing off), suggesting that some component(s) of this layer had been disrupted (van Waasbergen et al., 1996). However, none of the

mutant spores examined completely lacked this outer layer, and no significant differences were found between mutant and wild-type spores in terms of their germination properties or resistance to lysozyme, chloroform, ethanol, and heat. Genetic analysis of these non-oxidizing mutants led to the subsequent isolation and sequence analysis of a cluster of seven genes, the *mnx* genes, which appeared to be essential for Mn(II) oxidation (van Waasbergen et al., 1996). The most downstream gene in the *mnx* cluster encodes the putative Mn(II) oxidase, MnxG. Regions of this gene product share significant similarity with multicopper oxidases, a diverse group of proteins that use multiple copper ions to oxidize a variety of substrates, the majority of which are organic compounds (e.g., ascorbate, diphenolics, syringaldazine, etc.) but also Fe(II) (Solomon et al., 1996). Particularly conserved regions include those that are involved in copper binding, and small amounts of copper (1 μM) enhanced Mn(II) oxidation by SG-1 spores (van Waasbergen et al., 1996). Azide, a potent metalloenzyme inhibitor which bridges the Type 2 and Type 3 copper atoms of multicopper oxidases, has also been shown to inhibit Mn(II) oxidation by SG-1 spores (Rosson and Nealson, 1982). These findings support the idea that MnxG is directly involved in the oxidation of Mn(II).

The *mnxG* gene was cloned into the pTRXFus (Invitrogen) expression vector, allowing for overexpression of a thioredoxin-fusion protein in *E. coli*. SDS-PAGE

analysis of crude lysates revealed a protein of the appropriate size (138 + 16.5 kDa) in the insoluble fraction (data not shown). However, like the two other bacterial multicopper oxidases involved in Mn(II) oxidation (Brouwers et al., 2000), CumA and MofA, from *P. putida* GB-1 and *L. discophora* SS-1 respectively, no activity could be recovered from the heterologously expressed protein. This may be due to improper refolding of the expressed proteins, the absence of essential cofactors, or the requirement of additional proteins for activity. The fact that the Mn(II)-oxidizing activity of SG-1 outermost spore layer extracts could only be recovered at the stacking/resolving gel interface of low percentage SDS-PAGE gels (data not shown) suggests the requirement of a high-molecular-weight multi-protein complex for activity.

To further establish the role of MnxG in Mn(II) oxidation by SG-1 spores, we employed an immunological approach. Anti-MnxG antibodies, generated using expressed protein as the immunogen, were used in Western blot analysis of outermost layer extracts (Fig. 3). A protein of the expected size (~138 kDa) was detected in wild-type spores, indicating that it was in fact localized to the spore surface layer where the activity was localized. The fact that mutants with transposon insertions in *mnxA-F* exhibit the same non-oxidizing phenotype as those with insertions in *mnxG* suggested that the upstream insertions might simply be exerting a polar effect on

mnxG. To investigate this, various *mnx* mutants were also screened for the presence of MnxG via Western analysis, and MnxG was shown to be absent in each case, suggesting it may be the only Mnx protein directly involved in Mn(II) oxidation. The absence of MnxG (or a smaller truncated product) in spores of even the most downstream insertion mutant within the *mnx* operon (located at the 3' end of *mnxG*) suggests that, although most of the protein may be translated, it is most likely misfolded or mislocalized. There is also a putative copper-binding site (**HTFHLHGH**) near the C-terminus of MnxG that may be essential for proper folding, localization, and activity.

The localization of Mn(II)-oxidizing activity and the multicopper oxidase, MnxG, to the outermost layer of SG-1 spores led us to test spores of several other Mn(II)-oxidizing *Bacillus* isolates for the presence of such a layer (see Chapter 4). In every case, a significant amount of the activity was recovered in the supernatant following French press treatment, and SDS-PAGE analysis revealed complex, but unique, banding patterns relative to SG-1. This suggests that the presence of an outermost layer (exosporium) may be a common feature of Mn(II)-oxidizing spores. Future studies should reveal whether the Mn(II)-oxidizing proteins (and genes) of these *Bacillus* isolates are related. Ultimately, these studies may provide new insights

into the mechanism of bacterial Mn(II) oxidation as well as the biological function of this environmentally important process.

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TABLE 1. Gross chemical composition of isolated outermost layer and spore coat.

Component Analyzed	Outermost Layer % dry weight	Spore Coat % dry weight
Total	63.77	96.90
Protein (18 amino acids)	52.62	84.40
Polysaccharide	4.85	4.92
Glucosamine	2.98	2.88
Galactosamine	1.64	1.67
Glucose	0.13	0.22
Mannose	0.04	0.05
Galactose	0.04	0.05
Xylose	0.02	0.05
Lipid	6.30	7.58

TABLE 2. Amino acid analysis of outermost layer and spore coat.

	Outermost Layer		Spore Coat	
Constituent	% dry weight	Molar ratio[a]	% dry weight	Molar ratio[a]
Alanine	3.62	0.96	11.70	0.71
Arginine	3.70	0.44	3.41	0.36
Aspar-x	5.58	0.91	3.63	1.07
Glutam-x	8.39	1.23	9.05	0.74
Glycine	3.04	1.00	4.00	1.00
Histidine	1.07	0.15	5.43	0.18
Isoleucine	2.34	0.39	4.77	0.29
Leucine	3.94	0.65	5.06	0.46
Lysine	3.31	0.49	1.74	0.51
Methionine	1.42	0.20	3.15	0.14
Phenylalanine	4.67	0.60	4.99	0.47
Proline	1.94	0.38	6.94	0.43
Serine	2.25	0.49	6.54	0.44
Threonine	2.06	0.38	2.35	0.35
Tyrosine	2.65	0.30	6.25	0.45
Valine	2.64	0.50	5.39	0.54

[a] Glycine = 1.0.

TABLE 3. Fatty acid analysis of outermost layer and spore coat.

Fatty Acid	Vegetative cells (% of total fatty acids)	Outermost Layer (% of total fatty acids)	Spore Coat (% of total fatty acids)
br14:0	3.79	3.74	17.52
14:0	3.05	0.40	Trace
14:1	0.74	Trace	Trace
br15:0	21.75	35.00	29.90
15:0	43.80	21.10	14.31
br16:0	0.88	1.71	7.95
br16:1	6.49	7.76	Trace
16:0	0.61	0.95	10.45
16:1	2.57	2.03	Trace
br17:0	Trace	1.44	Trace
17:0	3.31	14.05	12.22
17:1	12.97	11.73	7.48
Total unsaturated	16.28	13.76	7.48
Total branched	32.91	49.65	55.37
Total saturated	50.77	36.50	36.98

Figure 1. Recovery of Mn(II)-oxidizing activity in the supernatant following French press-treatment of wild-type spores (left) but not in spores of a non-oxidizing mutant (right). Supernatants were incubated in HEPES buffer (pH 7.6) containing 100 μM $MnCl_2$, resulting in the formation of brown colloidal Mn oxides in the wild-type sample.

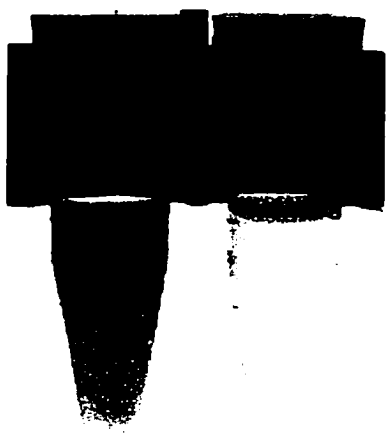
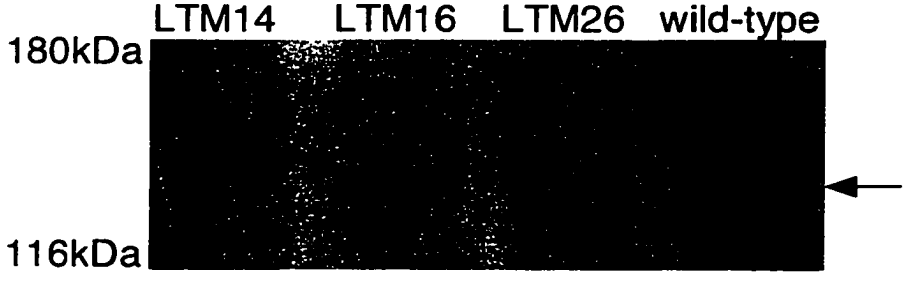


Figure 2. Transmission electron micrograph demonstrating the effect of French press-treatment on the ultrastructure of SG-1 spores. The ridged outermost layer surrounding the untreated spores (left) is partially removed by the French press (right). Remnants of the outer layer can be visualized by the patches of black Mn oxide precipitates still associtated with the stripped spores. Bars = 0.25 μM.

Figure 3. A Western blot of outermost layer extracts from spores of wild-type SG-1 and non-oxidizing mutants (LTM14, LTM16, LTM26) probed with antiserum generated to expressed-MnxG. A band of the expected size (~138 kDa) is present in wild-type extracts but absent in those of the mutants.



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CHAPTER IV

Enzymatic Manganese(II) Oxidation by Metabolically-Dormant Spores of Diverse *Bacillus* Species

ABSTRACT

Bacterial spores are renowned for their longevity, ubiquity, and resistance to environmental insults, but virtually nothing is known regarding whether these metabolically-dormant structures impact their surrounding chemical environments. In the present study, a number of spore-forming bacteria were isolated from coastal marine sediments which produce dormant spores that enzymatically oxidize soluble Mn(II) to insoluble Mn(IV) oxides. The highly charged and reactive surfaces of these metal oxides dramatically influence the oxidation and sorption of trace metals and organics in the environment. Prior to this study, the only other known Mn(II)-oxidizing spore-former was the marine *Bacillus* sp. strain SG-1, a model bacterium in which Mn(II) oxidation is believed to be catalyzed by a multicopper oxidase, MnxG. Phylogenetic analysis based on 16S rRNA and *mnxG* sequences obtained from 15 different Mn(II)-oxidizing spore-formers (including SG-1) revealed extensive diversity within the genus *Bacillus*, with organisms falling into several distinct clusters and lineages. In addition, active Mn(II)-oxidizing proteins of various sizes, as observed in SDS-PAGE gels, were recovered from the outer layers of purifief dormant spores of the isolates. These are the first active Mn(II)-oxidizing enzymes ever identified in spores or marine bacteria. Although extremely resistant to denaturation,

the activity of these enzymes was inhibited by azide and o-phenanthroline, consistent with the involvement of multicopper oxidases. Overall, these studies suggest that the commonly held view that bacterial spores are merely "inactive" structures in the environment should be revised.

INTRODUCTION

The uncanny resistance of bacterial spores to physical and chemical insults (e.g., heat, desiccation, radiation, oxidants, proteases, etc.) (Nicholson et al., 2000) enables them to persist in the environment for as many as thousands to millions of years without losing their capacity for germination and outgrowth (Cano et al., 1995; Gest and Mandelstam, 1987; Kennedy et al., 1994; Potts, 1994; Vreeland et al., 2000). In the dormant resting state, totally devoid of metabolic activity, spores are generally assumed to have a negligible impact on the chemistry of the environments that they inhabit. There are, however, known examples of how spores may influence the biology (biota) of a given habitat, such as the spore-associated insecticidal crystal proteins produced by *Bacillus thuringiensis* and *B. sphaericus* (Porter et al., 1993; Schnepf et al., 1998). Although a variety of enzymes are known to be associated with both the morphogenesis and germination of bacterial spores (Driks, 1999), to what

extent spore-associated enzymatic activities may actually influence their surrounding chemical environment is virtually unknown.

The only described example of bacterial spores that can significantly impact the distributions of heavy metals in the environment is the *Bacillus* sp. strain SG-1, isolated over 20 years ago from a shallow marine sediment off Scripps Pier (Nealson and Ford, 1980). Dormant endospores of this organism enzymatically catalyze the oxidation of soluble manganese(II) to highly insoluble Mn(III,IV) oxide precipitates on the spore surface, thereby becoming encased in a metal oxide shell (Rosson and Nealson, 1982). The highly charged and reactive surfaces of Mn oxides are known to dramatically influence chemical distributions in the environment, by oxidizing a wide array of organic and inorganic compounds as well as scavenging numerous heavy metals (e.g., Cu, Co, Cd, Zn, Ni, Pb) and radionuclides out of solution (Hem, 1978; Murray, 1975). SG-1 spores can increase the rate of Mn(II) oxidation, a thermodynamically favorable but kinetically slow reaction at neutral pH, by 4-5 orders of magnitude relative to abiotic rates (Hastings and Emerson, 1986). In addition, biogenic Mn oxides have been shown to have significantly greater surface area and trace metal adsorption capacity than abiotically produced Mn oxides (Nelson et al., 1999).

SG-1 has been studied as a model Mn(II)-oxidizing organism for many years (Francis and Tebo, 1999; Tebo et al., 1997), due in part to the remarkable stability of the Mn(II)-oxidizing activity over a wide range of environmental conditions, including: temperature (3-70°C), metal concentration (<nM to >mM), and ionic strength (freshwater to seawater). Molecular genetic studies of Mn(II) oxidation by SG-1 have revealed the involvement of a specific gene product, MnxG, which shares sequence similarity with multicopper oxidases (van Waasbergen et al., 1996). Members of this diverse family of proteins, including ascorbate oxidase, laccase, and ceruloplasmin, utilize copper ions of three spectroscopically distinct types as cofactors in the oxidation of a variety of substrates (Ryden and Hunt, 1993; Solomon et al, 1996). The recent identification of multicopper oxidase-like genes involved in Mn(II) oxidation in two other phylogenetically distinct proteobacteria, *Pseudomonas putida* GB-1 (Brouwers et al., 1999) and *Leptothrix discophora* SS-1 (Corstjens et al., 1997), suggests that these bacterial Mn(II) oxidases may represent a new functional class of multicopper oxidases. Recent spectroscopic studies have demonstrated that Mn(IV) minerals are the primary product of Mn(II) oxidation by SG-1 spores (Bargar et al., 2000), formed most likely through sequential one-electron transfers with a transient Mn(III) intermediate, consistent with the involvement of a multicopper oxidase.

Spore-forming *Bacillus* species are ubiquitous in the environment and can constitute a significant percentage of the total colony-forming bacteria in certain aquatic environments (20 to 40%) and sediments (up to 80%) (Bonde, 1981). Our laboratory has demonstrated that a considerable portion (17-33%) of the culturable spore-forming bacteria isolated from coastal surface sediments of Mission Bay and San Diego Bay, California produce Mn(II)-oxidizing spores (Lee and Tebo, unpublished). In addition, the heat-resistant fraction of the microbial populations in these sediments accounted for a large fraction of the total Mn(II)-oxidizing activity, suggesting that spores may be major catalysts of this process in situ. In the present study, we report for the first time that Mn(II)-oxidizing spore-formers represent a phylogenetically diverse group of organisms within the genus *Bacillus*, based on both 16S rRNA and MnxG (multicopper oxidase) sequences obtained from marine sediment isolates. Our results suggest not only that the capacity to produce metal-oxidizing spores may be widespread and evolutionarily important within this environmentally ubiquitous genus, but also that the extremely resilient Mn(II)-oxidizing spore enzymes have the potential to significantly impact the biogeochemical cycling of elements in aquatic sedimentary environments.

MATERIALS AND METHODS

Sample collection and strain isolation. Surface sediments were collected from San Diego Bay (SD), Mission Bay (MB), and Point Loma (PL) (San Diego, CA), diluted in sterile seawater, incubated at 80°C for 10 minutes, and spread onto Mn(II)-containing K plates (Rosson and Nealson, 1982). K medium contains 2.0 mg of peptone (Difco) per ml and 0.5 mg of yeast extract (Difco) per ml in sterile 75% seawater with both 20 mM HEPES (pH 7.6) and 100 μM $MnCl_2$ added after autoclaving. Mn(II)-oxidizing strains were isolated based on their ability to produce brown Mn oxide-encrusted colonies on plates. The presence of Mn oxides was confirmed using the colorimetric dye Leucoberbelin blue (Krumbein and Altman, 1973). Additional strains used in this study were the *Bacillus* sp. strain SG-1, *Bacillus subtilis* PY79, and 9 classical *Bacillus* strains obtained from the American Type Culture Collection (ATCC): *B. cereus* ATCC 1087, *B. circulans* ATCC 4513 (T), *B. firmus* ATCC 14575 (T), *B. licheniformis* ATCC 14580 (T), *B. marismortui* ATCC 700626, *B. megaterium* ATCC 14581 (T), *B. pumilus* ATCC 72, *B. thuringiensis* ATCC 35866, and *Halobacillus litoralis* ATCC 700076. These strains were tested for Mn(II) oxidation on K media made with either seawater or deionized water, depending on the origin of the strain.

DNA extraction, PCR, cloning, and sequencing. DNA was extracted from cultures using the DNeasy DNA extraction kit (Qiagen). For amplification of 16 rRNA genes, the primers 27F and 1492R (Lane et al., 1991) were used in a standard 30-cycle PCR using *Taq* polymerase and an annealing temperature of 50°C. For amplification of *mnxG* homologues from various *Bacillus* isolates, PCR primers were designed based on two of the copper-binding regions within the SG-1 sequence (van Waasbergen et al., 1996). The sequences are as follows: mnxGIF [5'-ACGCATGTCTTTCACTATCATGTTCAT-3']; and mnxGIR [5'-AAATAAGTGGTCATGGAAGAACCATGC-3']. The PCR program for *mnxG* amplification was 30 cycles of 94°(30 sec), 45°(30 sec), 60°(1 min), followed by one cycle of 72°C (15 min). PCR products were cloned into the vector pCR2.1 using a TOPO-TA cloning kit (Invitrogen, San Diego, CA). Plasmid DNA was purified using the Qiagen mini-prep kit (Qiagen) and both strands of the cloned PCR products were sequenced using an ABI 373A automated sequencer.

Phylogenetic analysis. 16S rRNA sequences were aligned manually using Sequencher™, compared to alignments generated using CLUSTALW and the Ribosomal Database Project (RDP) Sequence Aligner program, and both gaps and ambiguously aligned regions were removed. Phylogenetic trees were generated by neighbor-joining, using Jukes-Cantor corrected distances, or by maximum parsiomony

within the PAUP (version 4.0b3) software package. Derived MnxG amino acid sequences were aligned using CLUSTALW, and phylogenetic trees were constructed using neighbor-joining and parsimony methods within PAUP. Bootstrap analysis was used to estimate the reliability of phylogenetic reconstructions (1000 replicates).

Nucleotide sequence accession numbers. The 16S ribosomal RNA sequences of the 14 new *Bacillus* strains determined in the present study have been deposited in GenBank under the accession numbers AF326359-AF326373. The *mnxG* gene sequences have been deposited under the accession numbers AF326384-AF326397. The GenBank, EMBL, and DDBJ accession numbers for the 16S rRNA sequences used for comparison are as follows: *B. benzeovorans* (X60611), *B. cereus* (Z84581), *B. circulans* (D78312), *B. cohnii* (X76437), *B. firmus* (D16268), *B. globisporus* (X68415), *B. halmapalus* (X76447), *B. lentus* (D16272), *B. licheniformis* (X68416), *B. macroides* (AF157696), *B. marismortui* (AJ009793), *B. megaterium* (D16273), *B. methanolicus* (X64465), *B. pseudomoegaterium* (X77791), *B. pumilus* (AB020208), *B. simplex* (D78478), *B. sporothermodurans* (U49080), *B. subtilis* (X60646), *B. thuringiensis* (D16281), *Halobacillus litoralis* (X94558), *Listeria monocytogenes* (X98530), and *Virgibacillus pantothenticus* (D16275). The 16S rRNA sequences from the following environmental clones and isolates were also used: "*B. permians*" strain 2-9-3 (AF166093); BPC060 (AF15081) and BPC094 (AF15082);

LMG-19416 (AJ276808); strain HTE831 (AB010863); strain SB45 (AJ229238); and strain YKJ-11 (AF221062).

Isolation of Mn(II)-oxidizing acitivity from spores. Spores were purified from 1 L K cultures by the method of Rosson and Nealson (1982). The Mn(II)-oxidizing activity was isolated by passing purified spores through a French pressure cell six times at 20,000 psi. Stripped spores were then removed by centrifugation at 14,000 x g, and the supernatant, containing most of the activity, was recovered. Supernatants were assayed for Mn(II)-oxidizing activity by incubation in 10 mM HEPES (pH 7.6) containing 200μM $MnCl_2$ and observing the formation of brown Mn oxides. The effect of azide on Mn(II)-oxidizing activity was also tested.

SDS-PAGE analysis. Supernatants from French-press-treated spores of various isolates were mixed with 2X Laemmli buffer and proteins were separated by SDS-PAGE in standard 10% gels followed by staining with Coomassie blue for total protein (Laemmli, 1970). To assay for in-gel Mn(II)-oxidation activity, gels were first incubated in 0.5% Triton-X-100/10% glycerol for 30 min to remove SDS, and then incubated in 10 mM HEPES buffer (pH 7.6) containing 200 μM $MnCl_2$. Mn(II) oxidation was visualized by the formation of brown Mn oxide bands in gels after several hours of incubation. To determine the sensitivity of the Mn(II)-oxidizing

activity to copper chelators, gels were incubated in HEPES buffer (pH 7.6) containing o-phenanthroline (50 μM) for 15 minutes prior to the addition of 200 μM Mn(II).

RESULTS AND DISCUSSION

Although it has been recognized for nearly two decades that metabolically-dormant spores of the marine *Bacillus* sp. strain SG-1 can enzymatically oxidize soluble Mn(II) to Mn oxides, how widespread this environmentally-important capability is within the genus *Bacillus* has not previously been explored. In this study, a number of Mn(II)-oxidizing spore-formers were isolated from sediments collected within Mission Bay (MB), San Diego Bay (SD), and Point Loma (PL), near San Diego, California. Based on both phenotypic and phylogenetic characteristics, 14 different strains were chosen for further characterization, along with SG-1. Phylogenetic analysis of 16S rRNA genes obtained from these isolates revealed that the capacity to produce Mn(II)-oxidizing spores occurs in phylogenetically diverse organisms within the genus *Bacillus* (Fig.1). Many of the Mn(II)-oxidizing strains fell into distinct phylogenetic clusters with other Mn(II)-oxidizing isolates. Two of the three major clusters consist of closely related organisms isolated from different locations at different times. In fact, the five organisms within the SG-1 cluster were

isolated from four different environments over five different years, suggesting that this group of organisms may be particularly ubiquitous in marine sedimentary environments. Strains MB-11 and PL-26 appear to represent lineages distinct from the three major clusters and are only distantly related (<97%) to other sequences in the databases.

Four of the isolates (MB-1, -3, -7, and -9) clearly clustered (bootstrap value, 94%) with a group of organisms which are all known to be either moderately halophilic (growing optimally in media containing 3%-15% salt) or halotolerant (tolerating but not requiring high salt concentrations for growth). Phenotypically, many of the organisms within this cluster have been reported to be sufficiently different from the genus *Bacillus* to warrant classification into new genera, including *Halobacillus*, *Salibacillus*, *Virgibacillus*, and *Gracibacillus* (Heyndrickx et al., 1998; Spring et al., 1996; Waino et al., 1999). Database searches (BLAST and RDP) revealed that several of the most closely related sequences to MB-1, -3, and –7 were from "*B. permians*" strain 2-9-3, *Salibacillus* (formerly *Bacillus*) *marismortui*, and strain HTE831. Strain 2-9-3 was recently isolated from a brine inclusion within a reportedly 250 million-year-old salt crystal obtained from the Permian Salado (Vreeland et al., 2000). This organism can tolerate 20% NaCl and is very closely related (>99.5 %) to *Salibacillus marismortui*, a moderate halophile isolated from a

bottle of Dead Sea water collected in 1936 by Ben Volcani (Arahal et al., 1999; 2000). Strain HTE831 was recently isolated (Takami et al., 1999) from deep sea mud (1050m) off the south part of Japan and, consistent with its phylogenetic affiliation with this cluster, grows well on marine agar plates containing 2% to 23.4% NaCl. Similarly, the Mn(II)-oxidizing spore-formers (MB-1, -3, -7, and MB-9) exhibited growth on media containing 20% salt, supporting their phylogenetic affiliation within the "halo" cluster. A rather intriguing finding was that an environmental clone (LMG-19416) obtained from a biodeteriorated Austrian wall painting was 100% identical to the 16S rRNA sequence of MB-1 (1501 bp considered). It is tempting to speculate that the production of Mn(II)-oxidizing spores by LMG-19416 may have contributed to the "biodeterioration" of these paintings.

Another group of four Mn(II)-oxidizing spore-formers formed a tight phylogenetic cluster (the "PL-12 cluster") which also included the *Bacillus* strain YKJ-11 (99.9% identical to MB-5, 1488 bp considered), an organism isolated from the traditional Korean fermented seafood, Jeotgal. The overall PL-12 cluster also clearly grouped (bootstrap value, 100%) with a clade consisting of two hydrocarbon seep clones (BPC060 and BPC094) and a numerically-abundant isolate (SB45) obtained from rice paddy-associated anoxic bulk soil (Chin et al., 1999).

Based on the phylogenetic affiliations of the Mn(II)-oxidizing spore-formers described above, it is possible that *Bacillus* strains capable of producing Mn(II)-oxidizing spores may be present in a wide variety of environments, including hypersaline environments, coastal and deep sea sediments, hydrocarbon seeps, dry solid surfaces (e.g., wall paintings), and soils, where spore-forming *Bacillus* species are known to be particularly abundant. Direct evidence that this phenomenon is not limited to marine sedimentary environments comes from the fact that our laboratory has also isolated a number of Mn(II)-oxidizing spore-formers from Pinal Creek, an acid mine drainage-impacted, metal-contaminated stream near Globe, Arizona, in which Mn(II) is present at extremely high (0.5-1 mM) concentrations. In fact, several of the Pinal Creek organisms are closely related to organisms within the PL-12 cluster (>98% identity), while another strain clusters with PL-26 (data not shown), indicating that closely related Mn(II)-oxidizing *Bacillus* strains can, in some cases, be found in both terrestrial and marine environments. This also suggests that many of the marine sediment isolates may simply be halotolerant organisms, rather than true "marine" bacteria, which have been deposited in these sediments over time. This is supported by the fact that the capacity to grow in the presence of high salt was not restricted solely to organisms within the "halo" cluster. In fact, all of the Mn(II)-oxidizing spore-formers described in this study, except MB-11, were capable of growth on

media containing 10% salt and, surprisingly, organisms within the "PL-12" cluster were even capable of growth in the presence of 15 % salt.

The first genes ever shown to be involved in Mn(II) oxidation, the *mnx* genes, were previously identified in SG-1 using transposon mutagenesis (van Waasbergen et al., 1993; 1996). The most downstream gene in this gene cluster, *mnxG*, encodes a multicopper oxidase believed to be directly involved in Mn(II) oxidation. Biochemical evidence for the involvement of a multicopper oxidase in this process comes from the fact that the Mn(II)-oxidizing activity of SG-1 spores has been shown to be enhanced by low concentrations of copper (van Waasbergen et al., 1996) and inhibited by azide (Rosson and Nealson, 1982), a potent metalloprotein inhibitor which bridges the type 2 and type 3 copper atoms of multicopper oxidases. In order to determine whether the Mn(II)-oxidation-associated multicopper oxidase gene, *mnxG,* might also be involved in Mn(II) oxidation in these diverse isolates, these organisms were screened using PCR primers based on two of the copper-binding regions of MnxG (**H**VF**H**Y**H**VH and FF**H**D**H**L) expected to be highly conserved due to their functional roles. A ~900 bp region of *mnxG* was successfully amplified from all 14 isolates. Multiple sequence alignments based on the derived amino acid sequences of this region of MnxG (Fig. 2) revealed that this protein is highly conserved in these

phylogenetically *Bacillus* strains, with identities (relative to SG-1) ranging from 70 to 85% and similarities from 80 to 90%.

Phylogenetic trees based on the MnxG amino acid alignments (Fig. 3a) revealed the presence of three well defined clusters as well as three more distantly related sequences, suggesting that a similar copper-dependent molecular mechanism may exist in a wide variety of diverse *Bacillus* strains. For comparative purposes, a 16S rRNA-based tree was also generated for these same 15 organisms (Fig. 3b). The overall topologies of the respective trees were remarkably similar, suggesting that is unlikely that the *mnxG* gene has been horizontally transferred throughout the genus, but instead that it may be an evolutionarily and functionally important gene within these organisms. Despite the similarity between the MnxG- and 16S-rRNA-based trees, as with many functional genes (e.g., *rpoD*, *gyrB*, etc.), the phylogeny based on the *mnxG* gene product appears to provide even higher resolution in distinguishing closely related organisms. For example, based on MnxG sequences, the organisms within the SG-1 cluster appear to be more distantly related, possibly representing several distinct species.

To determine how widespread the ability to produce Mn(II)-oxidizing spores was within the genus *Bacillus*, a number of well-known strains were also tested for Mn(II) oxidation, including: *Bacillus subtilis* PY79, and ATCC strains of *B. cereus*, *B.*

circulans, *B. firmus*, *B. licheniformis*, *B. marismortui*, *B. megaterium*, *B. pumilus*, *B. thuringiensis*, and *Halobacillus litoralis*. Out of all of these strains, only *B. pumilus* produced spores that exhibited any Mn(II)-oxidizing activity, albeit this oxidation was relatively weak. In addition, *mnxG* was not PCR-amplified from any of these strains, although it cannot be totally ruled out that *mnxG* homologues are present in some of these strains but differ in the primer sites. Analysis of the complete *B. subtilis* genome confirmed that this organism does not possess a *mnxG* homologue. However, *B. subtilis* does possess a gene encoding a spore coat protein, CotA (Donovan et al., 1987), that appears to be a multicopper oxidase based on sequence similarity. This protein is roughly half the size of MnxG (65 kDa vs. 138 kDa) and is responsible for the brown pigment (which is not due to Mn oxides) associated with *B. subtilis* spores. Preliminary data from our lab indicate that CotA appears to have phenoloxidase activity (data not shown) which, by analogy to fungal laccases, may be responsible for pigment formation.

In order to explore the biochemical mechanism of Mn(II) oxidation in these diverse *Bacillus* isolates, SDS-PAGE analysis was used to compare the proteins which directly catalyze this reaction (Fig.4). It has been previously demonstrated that use of a French press is an effective method for physically removing the Mn(II)-oxidizing outermost layer from SG-1 spores while still retaining activity (see Chapter 3). This

method was applied to purified spores from all of the isolates and, in each case, a significant amount of Mn(II)-oxidizing activity was removed from the spores. The activity of the proteins was found to be inhibited by 1 mM azide, consistent with the involvement of a metalloprotein (e.g., multicopper oxidase). SDS-PAGE analysis of the spore surface proteins revealed the presence of active Mn(II)-oxidizing protein bands of various sizes in all of the isolates (Fig. 4), the first such proteins ever identified in spores or marine bacteria. There were, however, significant differences between various isolates in terms of overall protein profiles and the sizes of Mn(II)-oxidizing proteins. For six of the isolates (PL-26, not shown, and the SG-1 cluster), Mn(II)-oxidizing activity was only recovered in what appears to be a high-molecular-weight complex which barely enters the resolving gel, while for the remaining 9 isolates, single Mn(II)-oxidizing bands were present in gels, ranging in size from ~90 to 120 kDa. MB-11 was unique in that significant activity was present both in a high molecular weight complex and in a doublet of ~90 kDa, possibly indicating structural differences in the spore surface layer (either an exosporium or spore coat) of this organism relative to the other isolates. Overall, the relative sizes of the Mn(II)-oxidizing proteins from the *Bacillus* isolates described in this study correlated well with the phylogenetic groupings, indicating a clear link between phylogeny and spore physiology.

The size differences of the various Mn(II)-oxidizing proteins could be due to different-sized genes, posttranslational modifications (e.g., proteolysis, glycosylation), or the requirement of additional proteins (e.g., in a complex) for activity. In *Leptothrix discophora* SS-1, a 110-kDa Mn(II)-oxidizing protein has been consistently recovered in SDS-PAGE gels (Adams and Ghiorse, 1987; Boogerd and de Vrind, 1987), yet the underlying multicopper oxidase gene, *mofA*, encodes a predicted 174-kDa protein (Corstjens et al., 1997), suggesting partial cleavage/proteolysis of the full length polypepetide. In *Pseudomonas putida* GB-1, Mn(II)-oxidizing activity has only been recovered in the form of high-molecular-weight complexes (250-kDa and 180-kDa) in native gels (Okazaki et al., 1997), suggesting the requirement of several proteins, including the multicopper oxidase, CumA, for activity (Brouwers et al., 1999; de Vrind et al., 1998).

To determine whether the Mn(II)-oxidizing activity of the spore surface proteins was copper-dependent (as in SG-1), gels were incubated in Mn(II) buffer with the addition of the copper-chelator, *o*-phenanthroline, at a concentration (50 μM) well below that of Mn(II) (200 μM) (Fig. 4). The fact that the activity of all of the proteins was inhibited by this treatment, combined with the azide-inhibition of this activity in spore extracts, is consistent with the involvement of Cu-dependent oxidases (e.g., MnxG) in these phylogenetically diverse marine *Bacillus* isolates. As one might

expect of proteins located on the surface of bacterial structures designed to persist under extreme environmental conditions, the Mn(II)-oxidizing enzymes described in this study are extremely resilient. In fact, spores can be treated with heat (70 to 80°C), multiple freeze-thaw cycles, fixatives (e.g., UV, glutaraldehyde, etc), SDS, lysozyme, and reductants (to remove Mn oxides), with no significant loss of activity (de Vrind et al., 1986; Rosson and Nealson, 1982). These properties indicate that these spores are basically stable enzymatic catalysts for the oxidative precipitation of metals in the environment. This is supported by the fact that, within the coastal sediments from which many of the Mn(II)-oxidizing spore-formers were isolated, the *in situ* Mn(II)-oxidizing activity was shown to be due primarily to heat-resistant organisms (e.g, spores) and was inhibited by azide (i.e., multicopper oxidase-inhbitior) (Lee, 1994). Overall, the results of this study suggest that the commonly held view that bacterial spores are totally inactive structures in the environment should be revised.

ACKNOWLEDGMENTS

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Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences from Mn(II)-oxidizing spore-formers (blue), diverse representatives within the genus *Bacillus*, and the most closely related sequences in GenBank and RDP. Percentages of bootstrap support (>60%) from 1,000 replicates are indicated at the branch points. The strain designations of the Mn(II)-oxidizers are based on the location of isolation: MB, Mission Bay; PL, Point Loma; and SD, San Diego.

Figure 2. Alignment of the derived amino acid sequences from 15 *mnxG* genes. White letters on a black field indicate amino acids conserved across all 15 protein sequences. Conserved histidine residues within the putative copper-binding regions are highlighted by an asterisk. Sites with at least 80% identical residues are indicated by gray shading. Amino acid sequences were aligned with CLUSTALW and highlighted using a Microsoft Excel coloring Macro.

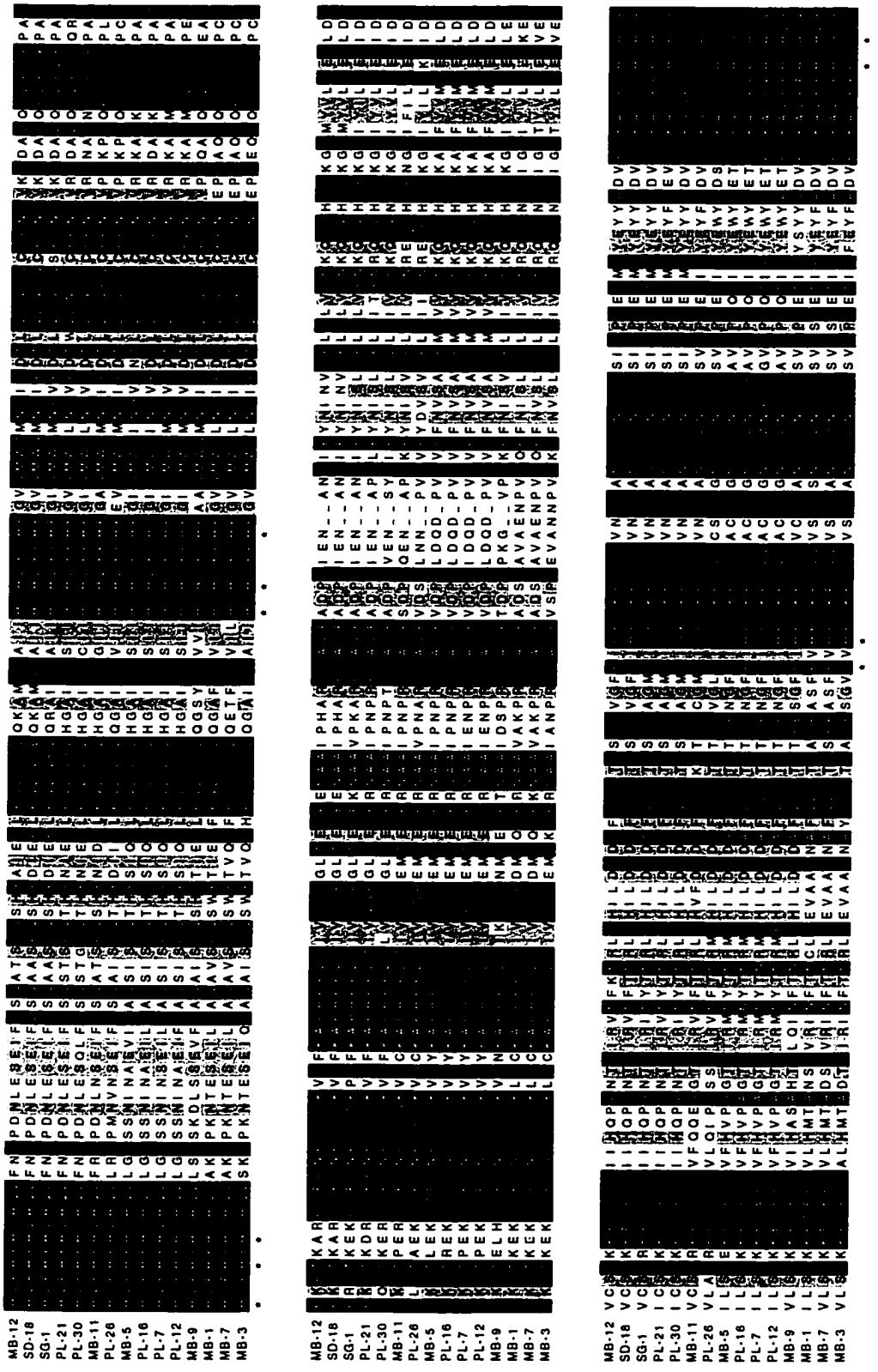


Figure 3. Unrooted neighbor-joining trees based on partial MnxG amino acid sequences and 16S rRNA sequences obtained from 15 Mn(II)-oxidizing spore-formers. Bootstrap values (>60%) are indicated at the supported nodes.

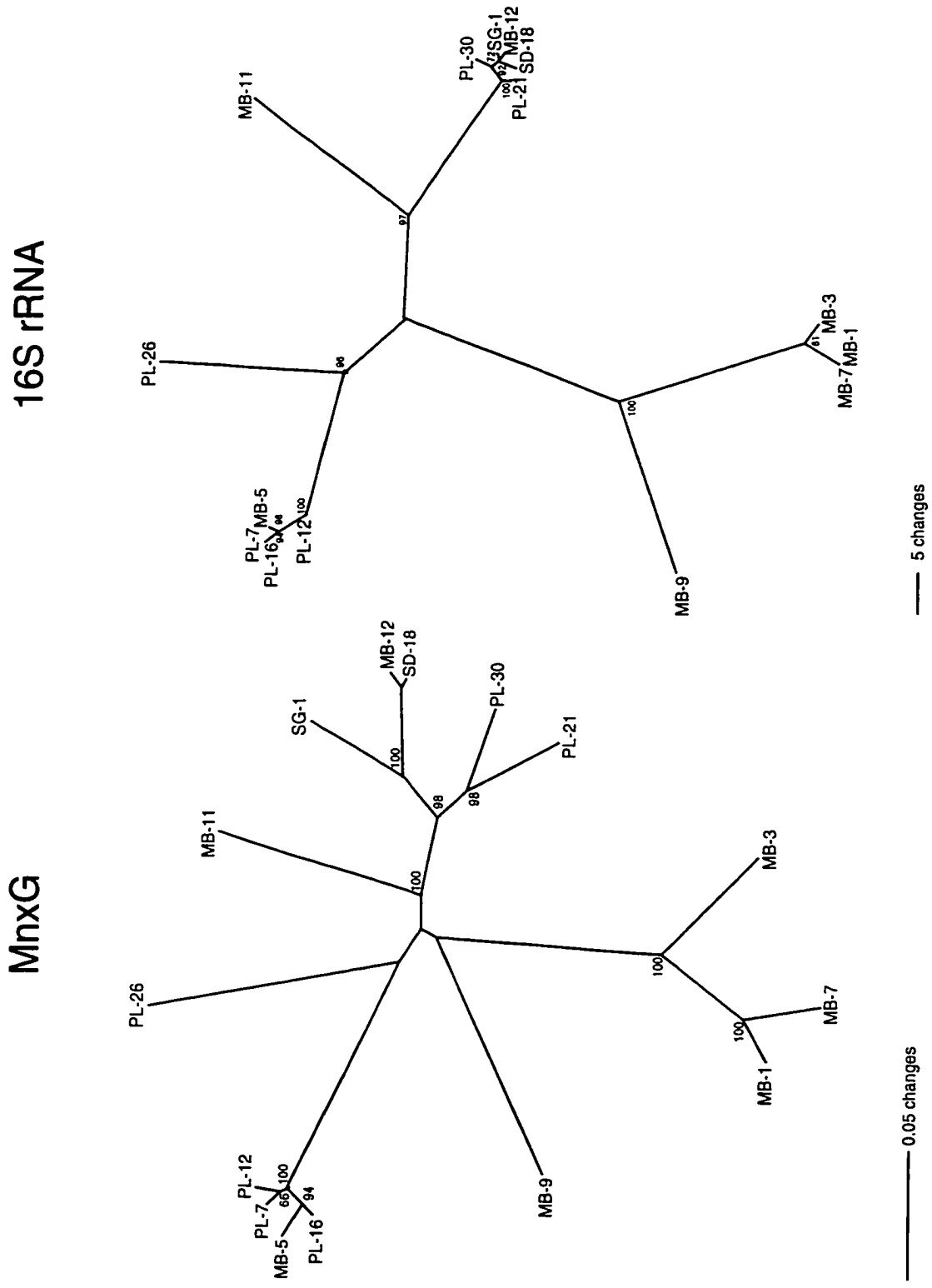
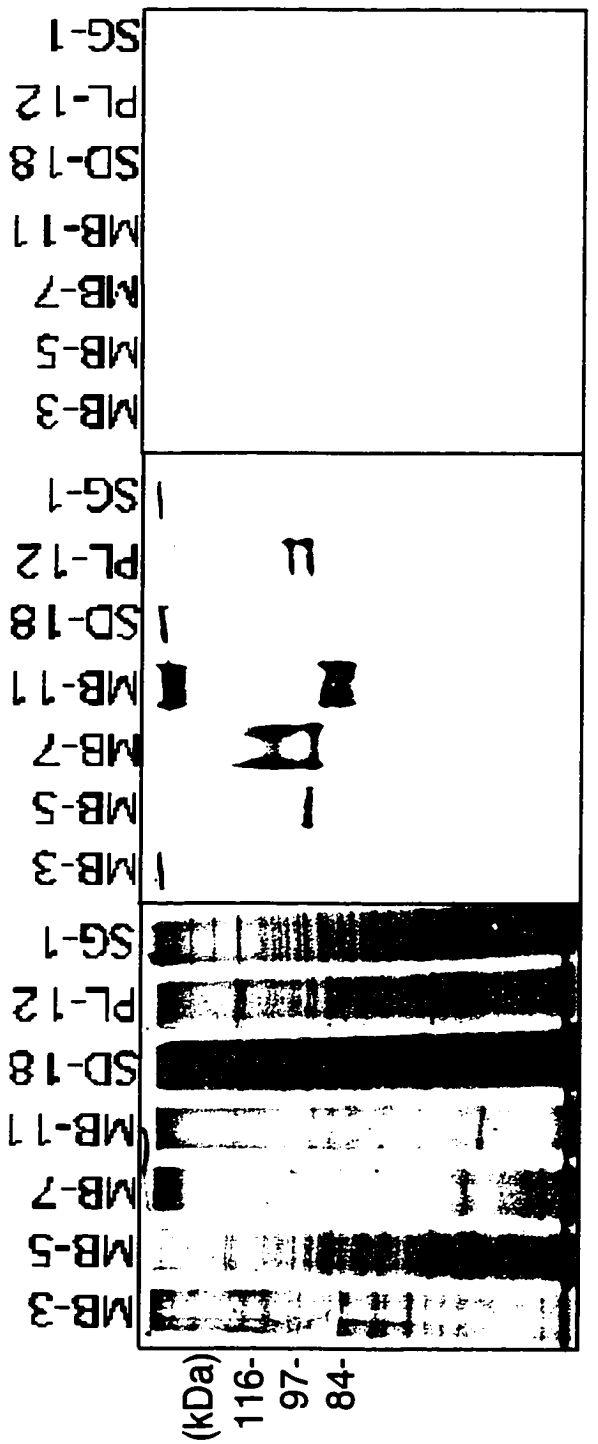


Figure 4. SDS-PAGE gels of outermost layer extracts from phylogenetically diverse Mn(II)-oxidizing spore-formers. Following elctrophoresis, identical gels were incubated in either Coomassie blue (left), Mn(II) buffer (center), or Mn(II) buffer containing the copper-chelator, *o*-phenanthroline (right). Active Mn(II)-oxidizing proteins were visualized as brown Mn oxide bands in gels incubated in Mn(II).



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CHAPTER V

Diversity of *cumA* Multicopper Oxidase Genes from Mn(II)-Oxidizing and Non-Oxidizing *Pseudomonas* Strains

ABSTRACT

Over the years, various *Pseudomonas* isolates have been reported to oxidize soluble manganese(II) to insoluble Mn(III,IV) oxides, but the underlying mechanism of catalysis has only recently started to emerge. Using transposon mutagenesis, a gene required for Mn(II) oxidation, designated *cumA*, was recently identified in *Pseudomonas putida* strain GB-1. This gene encodes a multicopper oxidase believed to be a key component of the outer membrane Mn(II)-oxidizing complex. In the present study, degenerate primers based on the copper-binding regions of the *cumA* gene product were used to PCR-amplify *cumA* gene sequences from a variety of *Pseudomonas* strains, including both Mn(II)-oxidizing and non-oxidizing strains. Alignment of the derived amino acid sequences demonstrated significant conservation not only of the copper-binding regions but of many other regions as well. The presence of highly conserved *cumA* gene sequences in several apparently non-oxidizing *Pseudomonas* strains suggests that this gene may not be expressed, may not be sufficient alone to confer the ability to oxidize Mn(II), or may have an alternative function in these organisms. Phylogenetic analysis of both CumA and 16S rRNA sequences revealed fairly similar topologies between the respective trees, including the presence of several distinct phylogenetic clusters. Overall, these results indicate that

both the *cumA* gene and the the capacity to oxidize Mn(II) occur in phylogenetically diverse *Pseudomonas* strains.

INTRODUCTION

Most of the manganese(II) oxidation which occurs in the environment is bacterially-mediated (Nealson et al., 1988; Tebo et al., 1997), yet the diversity of bacteria responsible for this activity and the underlying mechanisms of catalysis are poorly understood. Over the years, *Pseudomonas* strains capable of oxidizing Mn(II) have been isolated from a wide variety of environments, including soils, freshwater, seawater, water pipes, and even Mn nodules (Douka, 1977, 1980; Ehrlich, 1968; Ghiorse, 1984; Gregory and Staley, 1982; Jung and Schweisfurth, 1979; Schutt and Ottow, 1978). However, to date, the only well-characterized Mn(II)-oxidizing organisms within this genus are the closely related strains *P. putida* MnB1 and GB-1. Due to the ubiquitous nature of *P. putida* in the environment and the ease with which they can be grown, these strains have provided an excellent model system for studying bacterial Mn(II) oxidation.

Upon reaching stationary phase, *P. putida* strains MnB1 and GB-1 oxidize Mn(II) to Mn(III,IV) oxides which are precipitated on the cell surface, eventually

encrusting the organism. Previous studies suggested that MnB1 produces a soluble Mn(II)-oxidizing protein in late logarithmic/early stationary phase (DePalma, 1993; Jung and Schweissfurth, 1979). 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(II)-oxidizing activity of these factors, believed to be multi-protein complexes, is inhibited by the redox enzyme inhibitor azide as well as metal chelators, suggesting the involvement of a metal cofactor.

In order to identify genes involved in Mn(II) oxidation, transposon mutagenesis was used in both *P. putida* strains MnB1 and GB-1 (Caspi et al., 1998; de Vrind et al., 1998) to generate mutants which no longer oxidize Mn(II). In both studies, genes involved in the biogenesis and maturation of *c*-type cytochromes were found to be involved in Mn(II) oxidation. However, cytochromes alone are not believed to be sufficient for catalyzing this reaction. More recently, a gene encoding a multicopper oxidase, designated *cumA*, was reported to be essential for Mn(II) oxidation in GB-1 (Brouwers et al., 1999). This finding is consistent with the fact that multicopper oxidases have also been shown to be involved in Mn(II) oxidation in two other phylogenetically distinct organisms, the marine *Bacillus* sp. strain SG-1 (van Waasbergen et al., 1996) and the freshwater organism *Leptothrix discophora* SS-1

(Corstjens et al., 1997). In addition, small amounts of copper have been shown to enhance the rate of Mn(II) oxidation by all three organisms (Brouwers et al., 2000; Corstjens et al., 1997; van Waasbergen et al., 1996). Thus, *cumA* has been suggested to encode a Cu-dependent oxidase which is directly involved in Mn(II) oxidation.

The objective of this study was to assess the distribution and diversity of *cumA* genes within the genus *Pseudomonas*. In particular, a wide variety of *Pseudomonas* strains were screened both for their ability to oxidize Mn(II) and for the presence of the *cumA* gene. Phylogenetic analyses of both CumA and 16S rRNA sequences from both Mn(II)-oxidizing and non-oxidizing strains was used to determine how widespread the ability to oxidize Mn(II) is within this environmentally important genus.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and Mn(II) oxidation assays. The bacterial strains used in this study are listed in Table 1. Strains were maintained on *L. discophora* (LD) media (Boogerd and deVrind, 1987) containing 100 μM $MnCl_2$. The ability to oxidize Mn(II) was monitored by the formation of brown colonies on plates or visible Mn oxide formation in liquid cultures. The presence of Mn oxides was

confirmed using the colorimetric dye leucoberbelin blue (Krumbein and Altman, 1973).

DNA extraction, PCR, cloning, and sequencing. DNA was extracted from cultures using the QIAamp DNA extraction kit (Qiagen). The initial set of PCR primers was designed based on the two most distant copper-binding regions of the *P. putida* GB-1 *cumA* gene, and the sequences were as follows: CumAF [5'-ATCCATTGGCACGGCATCCGC-3']; and CumARdg [5'-TCCATRTGRTCRATSACRTGRCARTG-3']. Several internal primers were also designed to amplify *cumA* from additional *Pseudomonas* strains, and the sequences were as follows: CumAIdgFB [5'-TBGADATGGAYGGCGTGCC-3']; CumAIdgR2 [5'-TCGTTCTTGCCSARCARRTASGTRTCGGTGAA-3']; CumAIdg2B [5'-GAYGCCGGYAGCTACTGGTAYCACCC-3']; and CumAIdgR [5'-ACYTTGAARSYCATGCCRTGCARRTG-3']. The PCR program for *cumA* amplification was 30 cycles of 94°(30 sec), 45°(30 sec), 60°(1 min), using *Taq* polymerase (Roche). For amplification of 16 rRNA genes, the primers 27F and 1492R were used in a standard 30-cycle PCR. PCR products were cloned into the vector pCR2.1 using a TOPO-TA cloning kit (Invitrogen, San Diego, CA). Plasmid DNA was purified using the Qiagen mini-prep kit (Qiagen) and both strands of the cloned PCR products were sequenced using an ABI 373A automated sequencer.

Phylogenetic analysis. 16S rRNA sequences were aligned manually in Sequencher™ 3.1, compare to alignments generated using the Ribosomal Database Project (RDP) Sequence Aligner, and both gaps and ambiguously aligned regions were removed. Phylogenetic trees were generated by neighbor-joining, using Jukes-Cantor corrected distances, or by maximum parsiomony within the PAUP (version 4.0b3) software package. Derived CumA amino acid sequences were aligned using CLUSTALW, and phylogenetic trees were constructed using neighbor-joining and parsimony methods within PAUP. Bootstrap analysis was used to estimate the reliability of phylogenetic reconstructions (1000 replicates).

Nucleotide sequence accession numbers. The 16S ribosomal RNA sequences of the *Pseudomonas* strains determined in the present study have been deposited in GenBank under the accession numbers AF326374-AF326383. The 15 new *cumA* gene sequences have been deposited under the accession numbers AF326398-AF326412. The GenBank, EMBL, and DDBJ accession numbers of the 16S rRNA sequences used for comparison are as follows: *Escherichia coli* (AF233451), *P. aeruginosa* (Z76651), *P. alcaligenes* (Z76653), *P. alcalophila* (AB030583), *P. aureofaciens* (AF094722), *P. chlororaphis* (D86004), *P. flavescens* (U01916), *P. fluorescens* (D86001), *P. mandelii* (AF058286), *P. mendocina* (AF232713), *P. migulae* (AF074383), *P. pseudoalcaligenes* (Z76666), *P. putida* ATCC 12633

(AF094736), *P. putida* ATCC 17484 (D85993), *P. putida* mt-2 (D37924), *P. putida* MnB1 (U70977), *P. resinovorans* (AB021373), *P. stutzeri* JM300 (X98607), and *P. veronii* (AF064460). The accession numbers for the *cumA* sequences of *P. putida* GB-1 and *P. aeruginosa* PAO1 were AF086638 and AE004795, respectively.

Southern blot analysis. Chromosomal DNA from various strains was digested with restriction enzymes, separated by gel electrophoresis on 0.8% agarose gels, and transferred to nylon membranes. Digoxygenin-labeled probes were generated by using the DIG High Prime (Roche) random priming kit to label *cumA* PCR products obtained from *P. putida* MnB1 and *P. aeruginosa* ATCC15692. DNA was bound to the membranes by UV radiation, hybridized overnight with the DIG-labeled probe at 55°C, and washed in 2X SSC/0.1% SDS and 1X SSC/0.1% SDS at the same temperature, essentially by the method of Sambrook et al. (1989). Bound probe was detected using the chemiluminescent substrate CDP-star (Roche), according to manufacturer's instructions.

ABTS oxidation. To assay strains for laccase-like activity, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), a chromogenic susbstrate used for measuring laccase and peroxidase activities, was added to LD medium, with or without Mn(II), to a final concentration of 1 mM. The oxidation of this substrate resulted in the formation of a greenish/purple color on plates.

RESULTS

Phylogenetic diversity of organisms capable of Mn(II) oxidation.

In addition to the model Mn(II)-oxidizing strains, *P. putida* GB-1 and MnB1, several classical *P. putida* strains (ATCC 12633 and 33015) were also found to be capable of oxidizing Mn(II), although to a much lesser extent. Also, despite having been previously placed into separate biovars (DePalma, 1993) and, in several cases, having distinct colony morphologies and Mn(II)-oxidizing properties, several of Schweisfurth's isolates (MnB6, 11, 14, 18, 104) had 16S rRNA genes identical to that of MnB1. The finding that a variety of *P. putida* strains are capable of oxidizing Mn(II) is consistent with previous studies by DePalma (1993) suggesting that *P. putida* may be an important species involved in Mn(II) oxidation in the environment. The 16S rRNA sequence of the atrazine-degrading organism, *Pseudomonas* sp. strain ADP (de Souza et al., 1995), was fairly closely related (98.5%, 1450 bp considered) to some of these *P. putida* strains, but this organism did not oxidize Mn(II) on solid or liquid media.

A number of other environmentally important and well-characterized *Pseudomonas* species also did not appear to have the capacity to oxidize Mn(II),

including: *P. fluorescens* ATCC 13525, *P. syringae* pv. tomato PT23, *P. stutzeri* JM300, *P. aureofaciens* ATCC 13985, and *P. denitrificans* ATCC 13867. Although *P. aeruginosa* ATCC 15692 also did not oxidize Mn(II) under our experimental conditions, Brouwers et al. (1999) reported that logarithmic cultures of strain PAO1 could "in principle" oxidize Mn(II) but not reproducibly. The only other previously known species tested which oxidized Mn(II) to any extent was *P. chlororaphis* ATCC 9446.

Phylogenetic analysis of the 16S rRNA genes of a number of new Mn(II)-oxidizing strains (GB13, ISO1, ISO6, GP11, MG1, PCP, PCP2, and SI85-2B) isolated from a variety of environments revealed that the capacity to oxidize Mn(II) is not restricted only to close relatives of *P. putida* but actually appears to be quite widespread within the genus *Pseudomonas* (Fig.1), occurring in several distinct lineages. In addition, several of the Mn(II)-oxidzing isolates (e.g., GB13, PCP, ISO6, GP11) appear to be very closely related (>99.5% identity) to other *Pseudomonas* sequences in the databases (RDP and GenBank), suggesting that some of those organisms may also have the capacity to oxidize Mn(II). However, none of these strains have been tested for Mn(II) oxidation.

Amplification and sequence analysis of *cumA* genes.

The initial sets of PCR primers were designed based on two of the copper-binding regions (I**H**W**H**GIR and **HCH**VID**H**ME) of the deduced CumA amino acid sequence of GB-1, since these residues would be expected to be highly conserved due to their functional role. Although several primers were designed with varying degrees of degeneracy, the most effective primer combination was a non-degenerate forward primer (CumAF) and a degenerate reverse primer (CumARdg). These primers were used to successfully amplify *cumA* products of the expected size (~1056 bp) from 10 different *Pseudomonas* strains. Based on conserved regions of the 12 existing sequences (including *P. putida* GB-1 and *P. aeruginosa* PAO1), several additional internal primers were designed and used to amplify ~954 bp or ~810 bp regions of *cumA* from five additional strains which did not amplify with the other primers. Strain PCP2 was the only Mn(II)-oxidizing isolate for which no specific amplification occurred with any of the primer combinations. However, Southern blot analysis with a DIG-labeled *cumA* probe demonstrated the presence of a hybridizing band (data not shown).

Sequence analysis revealed that the *cumA* homologues ranged from 67% to 100% identity (at the DNA level) to the *P. putida* GB-1 sequence. The deduced CumA amino acid sequences also ranged from 67% to 100% identity, while the

percent similarities ranged from 81% to 100%, as expected from the translation of ambiguous codons into amino acid residues. The most divergent sequence was that of *P. aeruginosa* PAO1, whereas identical sequences were identified in *P. putida* MnB1 as well as the closely related strains MnB6, 11, 14, 18, 104 (data not shown). Alignment of the 17 derived CumA amino acid sequences (Fig. 2) revealed that certain regions of the protein were extremely highly conserved. As expected, the copper-binding regions were particularly conserved but, in addition, over 43% of the total amino acid residues were identical in all 17 sequences (>50% if *P. aeruginosa* PAO1 is excluded from the comparison).

Phylogenetic analysis of CumA sequences.

Phylogenetic trees based on all 17 CumA amino acid sequences revealed the presence of several distinct phylogenetic clusters (Fig. 3a). These included a "*P. putida* cluster", a "*P. fluorescens/syringae* cluster", and a group of four more divergent sequences (*P. stutzeri*, *P. aeruginosa*, and the Mn(II)-oxidizing isolates, SI85-2B and GP11). Relative to the CumA sequence of *P. putida* GB-1, sequences within the *P. putida* cluster shared an average of 97% identity/98% similarity; those within the *P .fluorescens/syringae* cluster shared 81% identity/ 88% similarity; and those within the third group shared 69% identity/82% similarity. The four *P. putida* strains formed a tight cluster, while the ADP sequence was slightly more divergent

(91% identity/93% similarity to GB-1). Five of the Mn(II)-oxidizing isolates fell within the *P. fluorescens/syringae* cluster, but only one of these organisms, strain PCP, would be classified as a strong Mn(II)-oxidizer. Strain ISO6, despite having an identical CumA sequence to that of PCP, is a relatively weak-to-moderate oxidizer, only oxidizing Mn(II) when streaked down into the agar, which suggests a preference for microaerobic conditions for Mn(II) oxidation. When streaked in this same manner, PCP initially oxidizes within the agar but eventually oxidizes uniformly on the surface of the plate as well. The other three Mn(II)-oxidizing isolates within this cluster (MG1, GB13, and ISO1), as well as *P. chlororaphis*, can all be classified as weak oxidizers while *P. fluorescens* is a non-oxidizer. *P. syringae*, also incapable of Mn(II) oxidation, appears to have a CumA sequence somewhat distinct from the *P. putida* and *P. fluorescens/syringae* clusters but, overall, clearly groups with the latter cluster (100% bootstrap value). The third group is composed of four distantly related sequences, with SI85-2B and *P. aeruginosa* clustering more closely with the other two distantly related phylogenetic clusters than with *P. stutzeri* or GP11.

For comparative purposes, a 16S rRNA phylogenetic tree was generated for the same 17 organisms from which CumA sequences were obtained (Fig. 3b). The overall topology of the 16S rRNA tree is fairly similar to the CumA tree, in that there are essentially two tight phylogenetic clusters and a group of more divergent

sequences which are not closely related to one another. One obvious difference, however, is that at the 16S rRNA level the PCP/ISO6 clade is less tightly associated with the *P. fluorescens/syringae* cluster, which is interesting since PCP and ISO6 are stronger oxidizers than the other Mn(II)-oxidizing strains within this overall cluster. The relationship between the four *P. putida* strains and strain ADP is also quite similar at the 16S rRNA level, with ADP being distinct from the tight cluster of *P. putida* strains. This distinction may reflect different physiological properties of strain ADP relative to *P. putida*, such as the rare ability to degrade atrazine (de Souza et al., 1995) as well as the inability to oxidze Mn(II). Finally, the relative relationships of *P. stutzeri*, SI85-2B, GP11, and *P. aeruginosa* appear to differ somewhat at the 16S rRNA. As can be seen in Fig.1, at the 16S rRNA level, strain PCP2 clusters more closely with *P. aeruginosa* than do any of the other Mn(II)-oxidizers, suggesting that this strain might also fall in a similar position within a CumA tree.

Alternative organic substrates for Mn(II)-oxidizing organisms.

Since all known multicopper oxidases are capable of oxidizing organic substrates (Solomon et al., 1996) and CumA shares significant sequence similarity with fungal laccases, in particular, both Mn(II)-oxidizing and non-oxidizing *Pseudomonas* strains were tested for the capacity to directly oxidize the synthetic laccase substrate, ABTS. All of the strong Mn(II)-oxidizers (*P. putida* MnB1 and GB-

1, PCP, PCP2, GP11, SI85-2B) oxidized the substrate, resulting in the formation of a greenish/purple color on plates (Fig. 4). However, none of the weak or non-oxidizing strains visibly oxidized the substrate. To further assess whether this activity was directly related to the ability to oxidize Mn(II), several non-oxidizing transposon mutants of *P. putida* MnB1 and GB-1 (Caspi et al., 1998; de Vrind et al., 1998), incapable of forming active Mn(II)-oxidizing complexes, were tested for ABTS oxidation (Fig.5). None of these mutants, including a *cumA* mutant and various *ccm* mutants, were able to oxidize ABTS, indicating a direct link between the Mn(II) oxidase and the oxidation of organic compounds.

DISCUSSION

Prior to this study, virtually nothing was known regarding the phylogenetic diversity of Mn(II)-oxidizing *Pseudomonas* species or the distribution of the Mn(II)-oxidation-associated gene, *cumA*, within this genus. The results of this study clearly indicate that the capacity to oxidize Mn(II) is not confined only to a few select *P. putida* strains but instead is quite widespread within the genus, occurring in several distinct lineages. In addition, the multicopper oxidase gene, *cumA*, also appears to be widely distributed within the genus *Pseudomonas*, occurring in both Mn(II)-oxidizing

and non-oxidizing strains. The similarity between the topologies of the CumA and 16S rRNA trees suggests that it is highly unlikely that the *cumA* gene has been horizontally transferred (e.g., via plasmids) throughout the genus *Pseudomonas*. Instead, it appears that the *cumA* gene may be an evolutionarily and perhaps functionally important gene in these organisms.

Although the overall phylogenetic clusters are fairly similar in the CumA and 16S rRNA trees, as with many functional genes, the phylogeny based on the *cumA* gene product may provide higher resolution than that based on the16S rRNA gene. For example, the relative phylogenetic placement of the Mn(II)-oxidizing isolates, PCP and ISO6, which group tightly within the core of the *P. fluorescens/syringae* cluster at the CumA level are more distantly related to this cluster at the 16S rRNA level. An explanation for this stems from the fact that the 16S rRNA sequences of strains PCP and ISO6 are almost identical to that of *P. putida* ATCC 17484 (biovar B). The taxonomic placement of this *P. putida* strain, as well as other biovar B strains, is somewhat controversial due to the fact that these strains have been reported to be more phenotypically similar to *P. fluorescens* than to classical *P. putida* (biovar A) strains. Our results are similar to those of Yamamoto and Harayama (1998) who found that strain ATCC 17484 clustered more closely with *P. fluorescens* than with *P. putida* (biovar A) strains based on *gyrB* and *rpoD* sequences. Based on such results, it

has been suggested that a reclassification of *P. putida* biovar B strains is necessary. Despite their phenotypic and phylogenetic differences, it is interesting that both biovar A and B strains of *P. putida* appear to share the capacity to oxidize Mn(II).

The presence of *cumA* gene sequences in various non-oxidizing *Pseudomonas* strains has a number of implications. For one, it is possible that the *cumA* gene product is simply not expressed and, thus, functionally inactive in the non-oxidizing strains. However, this seems rather unlikely considering how highly conserved this gene is in several of these organisms (e.g., ADP, *P. fluorescens*, etc.). The conservation of structural motifs (e.g., copper-binding regions) also suggests that these genes did not arise from duplications or related genes, since pseudogenes are under no selective pressure and would not be expected to maintain the structural elements needed for a functional protein (Scala and Kerkhof, 1999). To fully address this issue it will be necessary to employ techniques such as Northern analysis or reverse transcriptase-PCR to determine whether these genes are actually transcribed.

It should also be pointed out that the predicted CumA protein sequences of both *P. putida* GB-1 and *P. aeruginosa* PAO1 have predicted N-terminal signal peptides presumably involved in targeting the protein to the outer membrane (Brouwers et al., 1999). In addition, Brouwers et al. (1998) identified genes involved in a two-step protein secretion pathway essential for transporting the Mn(II)-oxidizing

factor across the outer membrane. Because our primers were designed based on the conserved copper-binding regions of CumA, no sequence data was obtained from the N-terminal region (~280 bp) of the 15 new *cumA* homologues described in this study. Thus, although seemingly unlikely, it cannot be totally ruled out that the inability of certain strains to oxidize Mn(II) could be due to differences in the signal peptides or the protein secretion pathways.

Since the Mn(II)-oxidizing factors isolated from *P. putida* GB-1 are believed to be multi-protein complexes, CumA may need to be directly associated with other proteins to have activity. Thus, it is possible that the *cumA* gene sequences from the non-oxidizing strains do encode functional proteins but that some other essential component of the complex is missing or inactive. Alternatively, it is possible that the non-oxidizing strains do, in fact, possess the genetic potential to oxidize Mn(II) but that they do so under different conditions (e.g., nutrient availability, O_2 level, metal concentration, etc.) than the other Mn(II)-oxidizing pseudomonads. To test whether *cumA* sequences from non-oxidizing *Pseudomonas* strains encode functional and properly localized proteins, attempts could be made to complement a *cumA* null mutant with *cumA* sequences from non-oxidizing strains. In addition, if *cumA* genes from various oxidizing strains were also tested in this way, it might be possible to

determine whether the differences in the degree or intensity of oxidation among strains are due to amino acid sequence differences or to some other factors (e.g., regulation).

Finally, it is possible that the *cumA* gene product has a different or alternative function in the non-oxidizing strains. In particular, the sequence similarity with fungal laccases suggested that, like all other known multicopper oxidases, CumA could be involved in the oxidation of organic substrates. Although phenolic compounds appear to be the primary substrates of laccases, these enzymes have also been shown to indirectly (e.g., non-specifically) oxidize Mn(II) to strongly oxidizing Mn(III) chelates which aid in the oxidation of lignin compounds (Archibald and Roy, 1992). In addition, a laccase was recently reported to directly oxidize Mn(II) to Mn(III) in the presence of the complexing agent Na-pyrophosphate (Hofer and Schlosser, 1999). Conversely, the Fe(II)-oxidizing multicopper oxidase, FET3 (from yeast), also has the capacity to oxidize organic compounds like *p*-phenylenediamine (K_m = 900 μM) but has a much higher affinity for Fe(II) (K_m = 2μM) (de Silva et al., 1997). A similar scenario exists for the multicopper ferroxidase ceruloplasmin found in vertebrate blood. Since a number of Mn(II)-oxidizing *Pseudomonas* strains were found to directly oxidize the synthetic laccase substrate, ABTS, a scenario analogous to that of the ferroxidases can be envisioned in which the metal is the primary substrate for CumA while the organic is a secondary, lower-specificity, substrate. The fact that

none of the weak Mn(II)-oxidizers visibly oxidized ABTS is consistent with this idea, in that an organism barely exhibiting Mn(II)-oxidizing activity would be expected to have correspondingly lower activity with the secondary organic substrate. A direct link between Mn(II)-oxidizing activity and ABTS oxidation was substantiated by the fact that the non-oxidizing transposon mutants of *P. putida* MnB1 and GB-1, incapable of forming active Mn(II)-oxidizing complexes, were also incapable of ABTS oxidation. Although an alternative function for *cumA* from non-oxidizing strains was not identified through these experiments, the range of possible substrates, activities, and functions of Mn(II)-oxidizing enzymes has been expanded.

The overlap in susbstrates of both laccases and Mn(II) oxidases is rather intriguing but it is unlikely that Mn(II) oxidases are simply bacterial laccases. For one, ABTS is just one of many phenolic substrates oxidized by laccases and it has yet to be determined how many of these compounds can be oxidized by Mn(II)-oxidizing organisms. In fact, Brouwers et al. (2000) recently reported that neither *P. putida* GB-1 nor *Leptothrix discophora* SS-1 were capable of directly oxidizing the model laccase substrate, syringaldazine. There has also only been one report of a fungal laccase directly oxidizing Mn(II) and it was only oxidized to Mn(III), whereas bacterial Mn(II) oxidation appears to lead primarily to the formation of Mn(IV) oxides (Bargar et al., 2000; Tebo et al., 1997). Although laccases have mainly been isolated and

studied from fungi and some plants (Thurston, 1994), several bacteria have now been shown to exhibit laccase-like phenoloxidase activity as well (Givaudan et al., 1993; Solano and Sanchez-Amat, 1999). The α-proteobacterium *Azospirillum lipoferum* produces an intracellular laccase involved in pigment formation (Givaudan et al., 1993), while the marine melanogenic γ–proteobacterium, *Marinomonas mediterranea* produces an extracellular polyphenoloxidase that is apparently not essential for pigment formation (Solano et al., 2000). Since all known bacterial Mn(II)-oxidizing proteins are cell surface-associated or extracellular, it is unlikely that the true function of the intracellular laccase of *A. lipoferum* is Mn(II) oxidation. In *M. mediterranea,* the first bacterial gene encoding an enzyme with laccase activity, designated *ppoA*, was recently identified using transposon mutagenesis (Solano et al., 2000). Although the biological function of this phenoloxidase is unknown, like the Mn oxidase of *P. putida*, the expression of this enzyme is induced during the stationary phase of growth (Fernandez et al., 1999). However, this organism does not appear to oxidize Mn(II) (Francis and Tebo, unpublished results). Finally, the 65-kDa spore coat protein, CotA, of *B. subtilis* which is responsible for spore pigmentation (Donovan et al., 1987), also appears to be a multicopper oxidase based on sequence similarity. This protein exhibits phenoloxidase activity but does not oxidize Mn(II) (Francis, Driks, and Tebo, unpublished results). Further studies of purified Mn(II)-oxidizing proteins, as well as

bacterial laccases, should reveal the relative affinities and specificities of these enzymes for metals and organic substrates.

Since highly conserved *cumA* sequences are present in a wide variety of phylogenetically diverse *Pseudomonas* strains, including strains incapable of Mn(II) oxidation, caution should be exercised if the *cumA* gene is used as a functional gene probe for "Mn(II) oxidation potential" in the environment. The primers used in this study were designed to amplify *cumA* from as many strains as possible and, thus, would not be appropriate for specifically detecting *cumA* from Mn(II)-oxidizing strains. However, based on the *cumA* sequences of the Mn(II)-oxidizing strains in this study, it may now be possible to design a suite of gene probes or PCR primers specific for *cumA* in Mn(II)-oxidizing pseudomonads. Linking the presence of Mn(II)-oxidation-associated multicopper oxidase genes (e.g., *cumA*, *mnxG*, *mofA*) with bacterial Mn(II) oxidation in the environment will be essential for establishing the importance of these Cu-dependent enzymes in nature.

ACKNOWLEDGMENTS

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TABLE 1. Mn(II)-oxidizing and non-oxidizing *Pseudomonas* strains used in this study

Organism	Strain	Origin of new Mn(II)-oxidizing isolates	Mn(II) Oxidation*	Source
Pseudomonas sp.	GB13	Sediments, Green Bay, WI	+	L. Stein
Pseudomonas sp.	GP11	Pulpmill Effluent, Grande Prairie, Alberta, Canada	+++	This study
Pseudomonas sp.	ISO1	*Metallogenium* particles from Horsetooth Reservoir, Fort Colllins, CO	+	L. Stein
Pseudomonas sp.	ISO6	"	++	L. Stein
Pseudomonas sp.	MG1	"	+	L. Stein
Pseudomonas sp.	PCP	Pinal Creek sediments, Globe, AZ	+++	This study
Pseudomonas sp.	PCP2	Pinal Creek sediments, Globe, AZ	+++	This study
Pseudomonas sp.	SI85-2B	Oxic/anoxic interface, Saanich Inlet, B.C.	+++	This study
P. putida	GB-1		+++	J.P. de Vrind
P. putida MnB1	ATCC 23483		+++	ATCC
P. putida	ATCC 12633		+	ATCC
P. putida mt-2	ATCC 33015		+	ATCC
P. chlororaphis	ATCC 9446		+	ATCC
P. aeruginosa	ATCC 15692		-	ATCC
P. aureofaciens	ATCC 13985		-	ATCC
P. denitrificans	ATCC 13867		-	ATCC
P. fluorescens	ATCC 13525		-	ATCC
P. stutzeri	JM300		-	B. Ward
P. syringae pv. tomato	PT23		-	D. Cooksey
Pseudomonas sp.	ADP		-	D. Crowley

*Relative Mn(II) oxidation on plates: +, weak or variable; ++, moderate; +++, strong

Figure 1. Neighbor-joining phylogenetic tree of 16S rRNA sequences from Mn(II)-oxidizing *Pseudomonas* strains (blue), non-oxidizing strains (red), and other closely related sequences in GenBank and RDP which were not tested for Mn(II) oxidation (black). Percentages of bootstrap support (>60%) from 1000 replicates are indicated at the branch points.

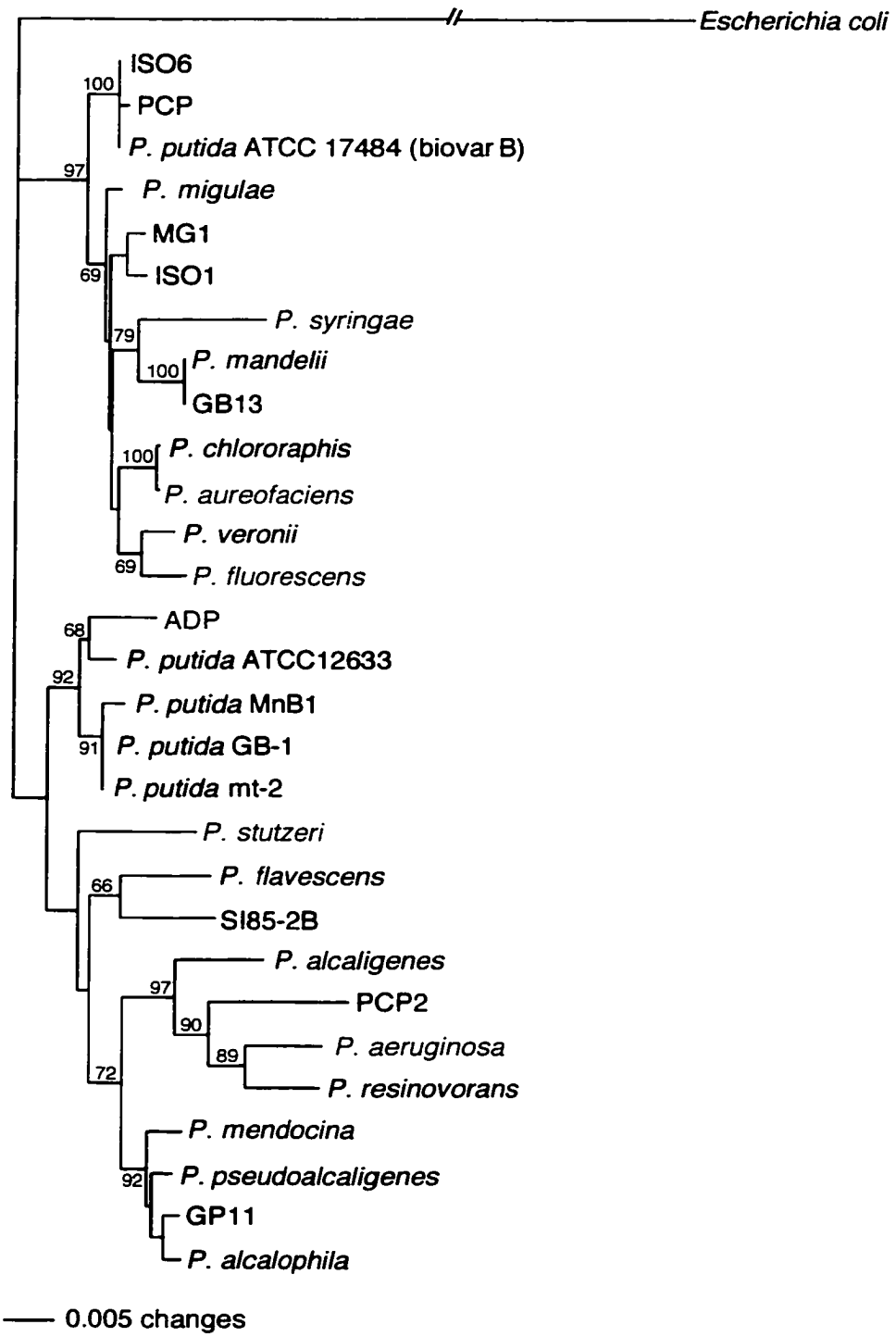


Figure 2. Alignment of the derived amino acid sequences from 17 *cumA* genes. White letters on a black field indicate amino acids conserved across all 17 protein sequences. Conserved histidine residues within the putative copper-binding regions are highlighted by an asterisk. Sites with at least 80% identical residues (14 out out of 17) are indicated by gray shading. Horizontal brackets correspond to the conserved regions targeted by our PCR primers. Amino acid sequences were aligned with CLUSTALW and highlighted using a Microsoft Excel coloring Macro.

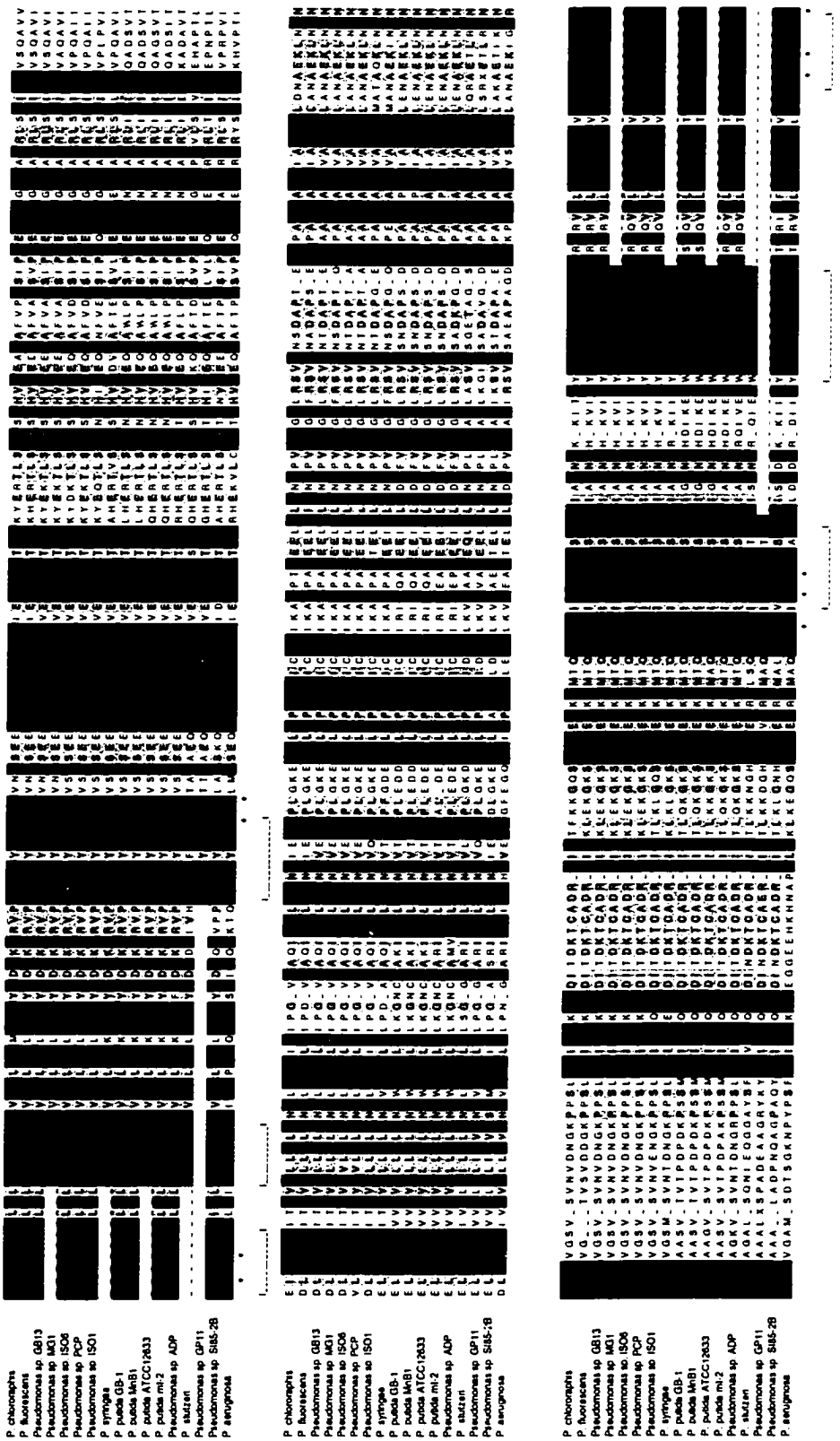


Figure 3. Unrooted neighbor-joining trees based on partial CumA amino acid sequences and 16S rRNA sequences from 17 *Pseudomonas* strains, including Mn(II)-oxidizing (blue) and non-oxidizing (red) strains. Bootstrap values (>60%) are indicated at the supported nodes.

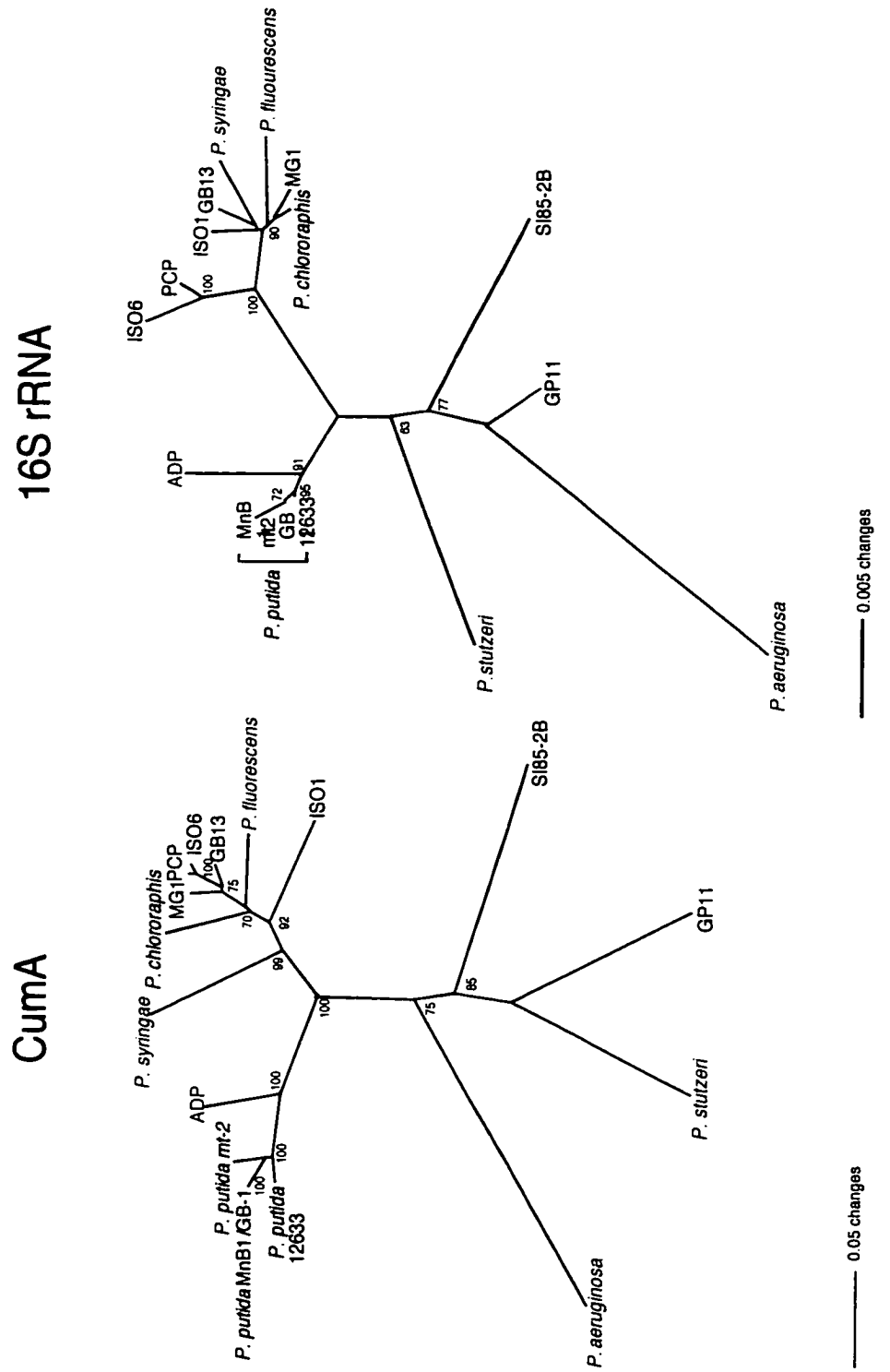


Figure 4. Oxidation of Mn(II) and the synthetic laccase substrate, ABTS, by diverse *Pseudomonas* strains. Strains were streaked on LD media containing either Mn(II) (upper row) or ABTS (lower row). Mn(II) oxidation results in the formation of brown Mn oxides on colonies, while ABTS oxidation results in the formation of a diffusible purplish/green product.

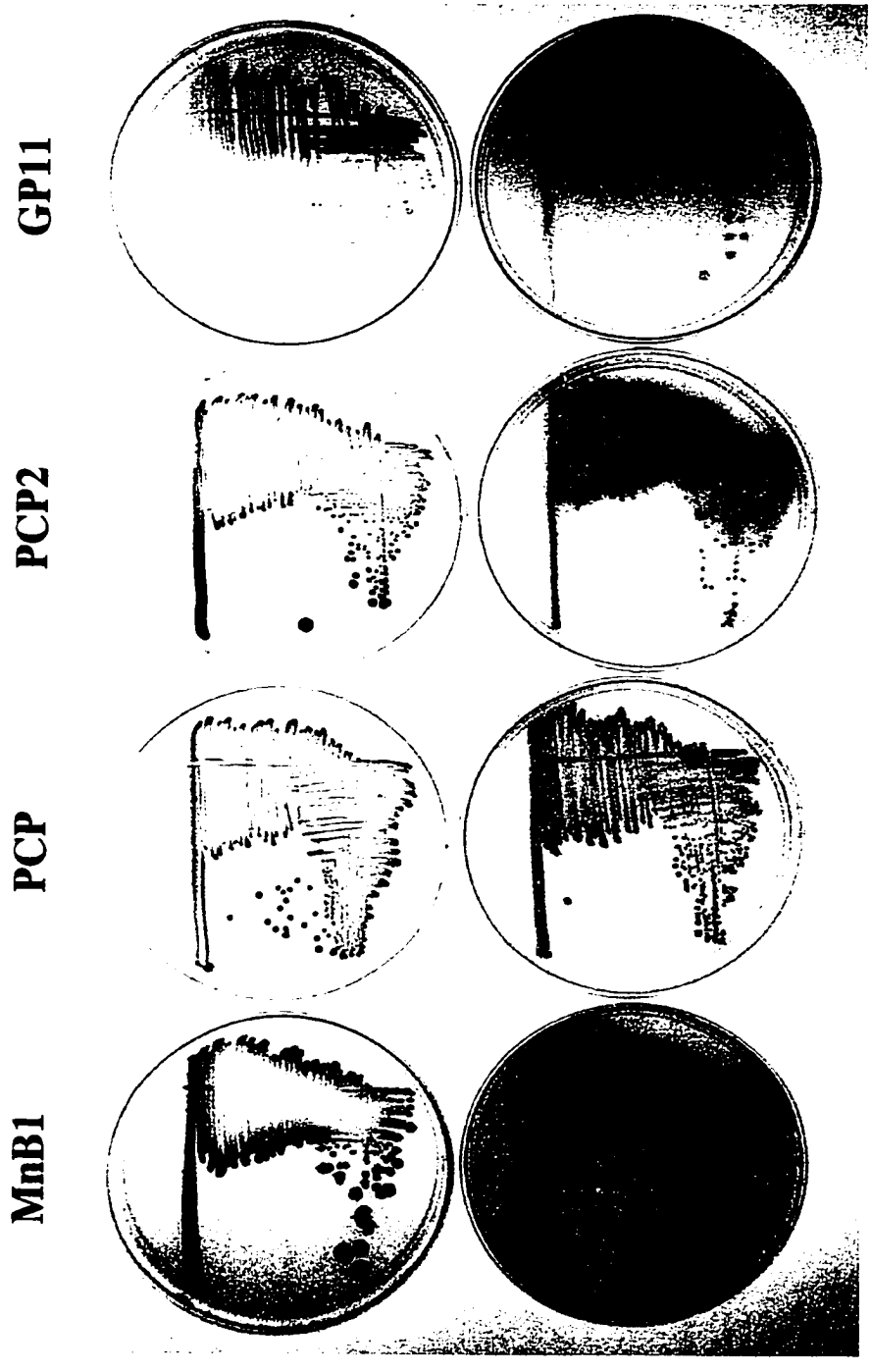
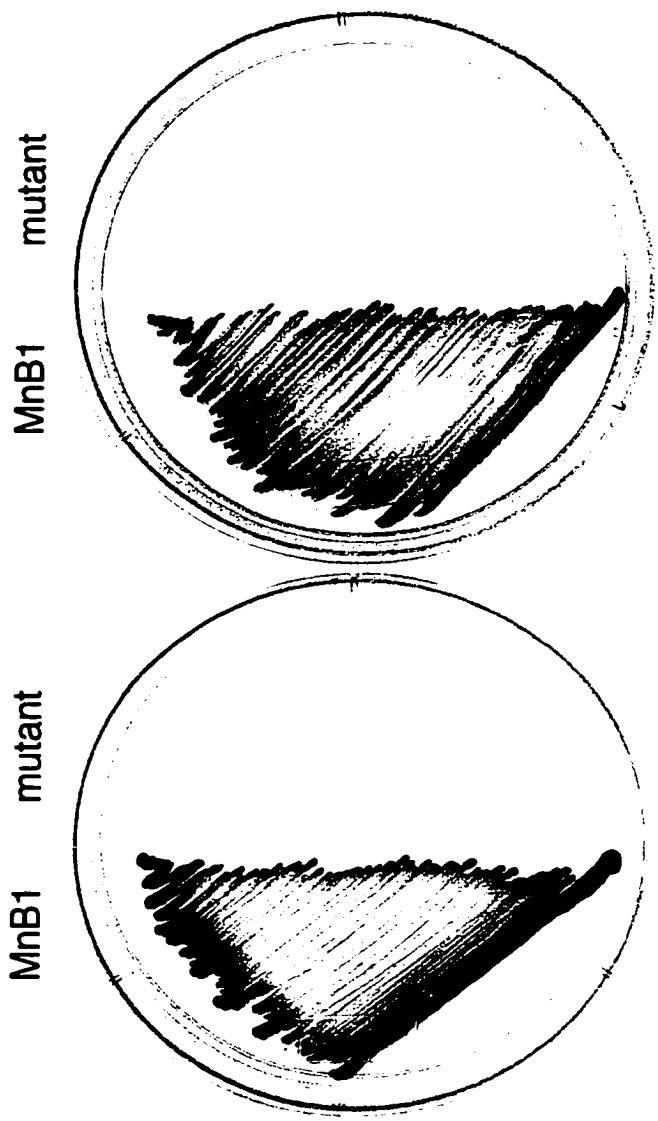


Figure 5. *P. putida* MnB1 and a non-oxidizing MnB1 mutant streaked on plates containing Mn(II) or ABTS. The mutant is incapable of oxidizing both substrates and remains opaque on plates.



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CHAPTER VI

Enzymatic Manganese(II) Oxidation by

a Marine α-Proteobacterium

ABSTRACT

A yellow-pigmented marine bacterium, designated strain SD-21, was isolated from surface sediments of San Diego Bay, CA, based on its ability to oxidize soluble Mn(II) to insoluble Mn oxides. 16S rRNA analysis revealed that this organism was most closely related to members of the genus *Erythrobacter*, aerobic anoxygenic phototrophic bacteria within the α-4-*Proteobacteria*. SD-21, however, has a number of distinguishing phenotypic features relative to *Erythrobacter* species, including the ability to oxidize Mn(II). During logarithmic phase of growth, this organism produces Mn(II)-oxidizing factors of ~250-kDa and 150-kDa that are heat labile and inhibited by both azide and o-phenanthroline, suggesting the involvement of a metalloenzyme. Although the expression of the Mn(II)-oxidase was not dependent on the presence of Mn(II), higher overall growth yields were reached in cultures incubated with Mn(II) in the culture medium. In addition, the rate of Mn(II) oxidation appeared to be slower in cultures grown in the light. This is the first report of Mn(II) oxidation within the alpha-4-*Proteobacteria* as well as the first Mn(II)-oxidizing protein identified in a marine Gram-negative bacterium.

INTRODUCTION

The oxidation of soluble manganese(II) to insoluble Mn(III,IV) oxids and oxyhydroxides is an environmentally important process, because the solid phase products oxidize a variety of organic and inorganic compounds (e.g., humic substances, Cr(III), Fe(II)), scavenge a variety of metals (e.g., Cu, Co, Cd, Zn, Ni, Pb), and serve as electron acceptors for anaerobic respiration. In most environments, Mn(II) oxidation is believed to be bacterially-mediated (Nealson et al., 1988). Over the years, Mn(II)-oxidizing bacteria have been isolated from a wide variety of environments, including marine and freshwaters, soils, sediments, water pipes, Mn nodules, and hydrothermal vents (Douka, 1977, 1980; Ehrlich, 1968; Ghiorse, 1984; Gregory and Staley, 1982; Juniper and Tebo, 1995; Jung and Schweisfurth, 1979; Nealson, 1978; Schutt and Ottow, 1978). Phylogenetically, Mn(II)-oxidizing bacteria appear to be quite diverse, with all isolates analyzed to date falling within either the low G+C Gram-positive, the actinobacteria, or α-, β-, and γ- *Proteobacteria* branches of the domain *Bacteria* (Tebo et al., 1997).

The most well-characterized Mn(II)-oxidizing bacteria are the *Bacillus* sp. strain SG-1, *Leptothrix discophora* SS-1, and *Pseudomonas putida* strains MnB1 and GB-1. Although distantly related phylogenetically, enzymes related to multicopper

oxidases appear to be involved in Mn(II) oxidation in all of these organisms (Brouwers et al., 1999; Corstjens et al., 1997; van Waasbergen et al., 1996). Multicopper oxidases are a diverse family of proteins that utilize multiple copper ions as cofactors in the oxidation of a wide variety of substrates (Solomon et al., 1996). In all the model systems, Mn(II)-oxidizing activity is also inhibited by azide (Boogerd and de Vrind, 1987; Okazaki et al., 1997; Rosson and Nealson, 1982), a potent inhibitor of multicopper oxidases, and stimulated by the presence of copper (Brouwers et al., 1999, 2000; van Waasbergen et al., 1996). These findings suggest the possibility of a universal mechanism for bacterial Mn(II) oxidation which is dependent on copper as an essential enzymatic cofactor.

Relative to the model Mn(II)-oxidizing organisms described above, very little is known regarding the mechanisms of Mn(II) oxidation within the α-*Proteobacteria*. Despite numerous reports of Mn(II) oxidation by various prosthecate bacteria (e.g., *Pedomicrobium*, *Hyphomicrobium*, *Caulobacter*, etc) within the α-*Proteobacteria* (Gebers, 1981; Ghiorse, 1984; Ghiorse and Hirsch,1982; Gregory and Staley, 1982; Sly et al., 1988; Tyler, 1970), few studies have directly addressed the biochemical mechanism(s) of Mn(II) oxidation in these organisms (Ghiorse and Hirsch, 1979, 1982). One recent study, however, demonstrated that Mn(II) oxidation by *Pedomicrobium* sp. ACM 3067 appears to be catalyzed by a copper-dependent

enzyme (Larsen et al., 1999), consistent with the possible involvement of a multicopper oxidase in this organism. However, no further purification of the Mn(II)-oxidizing enzyme or identification of the gene(s) involved has been reported. Another Mn(II)-oxidizing strain, SI85-9A1, is a novel marine α-proteobacterium which possesses genes for both the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Caspi et al., 1996). Although this was the first report of RubisCO genes in a Mn(II)-oxidizing bacterium, the molecular and biochemical mechanisms of Mn(II) oxidation in this organism have yet to be explored. Considering the abundance and diversity of α-proteobacteria found in the marine environment (Giovannoni and Rappé, 2000), it is important to determine both the diversity of organisms capable of Mn(II) oxidation within this group as well as the mechanisms underlying this environmentally important process.

In the present study, we describe the isolation and characterization of an organism, strain SD-21, which has a number of features that make it an attractive candidate as a new model Mn(II)-oxidizing α-proteobacterium.

MATERIALS AND METHODS

Sample collection and strain isolation. Surface sediments were collected from San Diego Bay and Mission Bay (San Diego, CA), diluted in sterile seawater, and spread onto K plates (Rosson and Nealson, 1982) containing 100 μM $MnCl_2$. Mn(II)-oxidizing strains were isolated based on their ability to produce brown Mn oxide-encrusted colonies on plates. The presence of Mn oxides was confirmed using the colorimetric dye Leucoberbelin blue (LBB) (Krumbein and Altman, 1973). Additional strains used in this study were *Erythrobacter litoralis* ATCC 700002 (T), *Erythrobacter longus* Och101 ATCC 33941 (T), and a yellow-pigmented Mn(II)-oxidizing *Sphingomonas* strain isolated from pulpmill effluent.

Physiological characterization. The growth temperature range of strain SD-21 was determined by incubating 5 ml K cultures over a range of temperatures (4°C, 12°C, 18°C, 25°C, 30°C, 37°C, and 42°C) for two weeks in the dark, and measuring the optical densities (600 nm) in a Perkin-Elmer spectrophotometer. The pH range for SD-21 growth was determined in K medium ranging from pH 5 to 9. The salt tolerance/requirement was determined by incubating SD-21 in K media made with artificial sea water containing a range of NaCl concentrations (0 to 15%). For pigment analysis, bacterial pellets (0.2g wet wt.) of dark-grown cultures of SD-21,

Erythrobacter longus, *Erythrobacter litoralis*, and the *Sphingomonas* isolate, were extracted with 2 ml of acetone/methanol (7:2 vol/vol). Absorption spectra (200 to 900 nm) were then obtained using a Perkin-Elmer spectrophotometer.

Growth experiments. Strain SD-21 was grown in 1 L flasks containing 500 ml of K media, on an orbital shaker (150 rpm), in the presence and absence of both Mn(II) and light. The optical densities (600 nm) of duplicate cultures were measured at 12 h intervals for 7 days. The production of Mn oxides in cultures was quantified spectrophotometrically with LBB (620 nm) relative to a standard curve of $KMnO_4$ as described previously (Lee and Tebo, 1994). The effect of Mn oxides on the optical densities of Mn(II)-grown cultures was determined by re-measuring the OD600 of cultures after removal of the oxides with ascorbic acid.

SDS-PAGE and analysis of Mn(II)-oxidizing activity. Cell suspensions (in 10 mM HEPES pH 7.6) were passed through a French press cell three times at 20,000 psi followed by centrifugation for 10 min at 14,000 x g to remove cell debris. Cell lysis was confirmed microscopically. Cell-free extracts were assayed for Mn(II)-oxidizing activity in 10 mM HEPES containing 200 μM Mn(II), followed by LBB detection. The effect of the potential enzyme inhibitors, SDS and azide, on cell-free activity was also determined. Supernatants were mixed with 2X Laemmli buffer and run in 10% SDS-polyacrylamide gels in a mini-Protean II (Bio-Rad) electrophoresis

unit under standard condtions (Laemmli, 1970). For staining of total protein, gels were incubated in Coomassie blue. To assay for in-gel Mn(II) oxidation activity, gels were first incubated for 30 min in 10% glycerol/0.1% Triton-X-100 to remove SDS, followed by incubation in 10 mM HEPES (pH 7.6) buffer containing 200 μM $MnCl_2$. Mn(II)-oxidizing activity was visualized by the formation of brown Mn oxide bands in gels after several hours of incubation. The temperature stability of the Mn(II)-oxidizing protein was determined by incubating cell-free extracts at room temperature, 37°C, 42°C, 45°C, 55°C, and 95°C for 15 min prior to running the gels. The sensitivity of the Mn(II)-oxidizing activity to copper chelators was assayed by incubating gels in HEPES buffer (pH 7.6) containing o-phenanthroline for 15 minutes prior to the addition of 200 μM Mn(II).

DNA extraction, PCR, cloning, and sequencing. DNA was extracted from cultures using the DNeasy DNA extraction kit (Qiagen). 16 rRNA genes were amplified using the primers 27F and 1492R in a standard 30-cycle PCR using Taq polymerase (Roche) with an annealing temperature of 50°C. PCR products were cloned into the vector pCR2.1 using a TOPO-TA cloning kit (Invitrogen, San Diego, CA). Plasmid DNA was purified using the Qiagen mini-prep kit (Qiagen), and both strands of the cloned PCR products were sequenced using an ABI 373A automated sequencer.

Phylogenetic analysis. 16S rRNA gene sequences were aligned manually using Sequencher™ 3.1, compared to alignments generated using CLUSTALW and Ribosomal Database Project (RDP) Sequence Aligner, and edited to remove ambiguously aligned regions and gaps. Phylogenetic trees were generated by neighbor-joining, using Jukes-Cantor corrected distances, or by maximum parsimony within the PAUP (version 4.0b3) software package. Bootstrap analysis was used to estimate the reliability of phylogenetic reconstructions (1000 replicates).

Nucleotide sequence accession numbers. The 16S ribosomal RNA sequences of SD-21 and MB-16 determined in this study have been deposited in GenBank under the accession numbers AF325445 and AF325446, respectively. The GenBank, EMBL, and DDBJ accession numbers for the 16S rRNA sequences used for comparison are as follows: *Acidiphilium cryptum*, D30773; *Agrobacterium tumefaciens*, D14500; *Azospirillum lipoferum*, X79730; *Beijerinckia indica*, M59060; *Caulobacter crescentus*, AJ227757; *Citromicrobium bathyomarinum*, Y16267; *Erythrobacter litoralis*, AB013354; *Erythrobacter longus*, L01786; *Erythromicrobium ramosum*, X72909; *Escherichia coli*, M24828; *Magnetospirillum gryphiswaldense*, Y10109; *Methylobacterium extorquens*, D32224; *Paracoccus denitrificans*, X69159; *Pedomicrobium manganicum*, X97691; *Porphyrobacter neustonensis*, L01785; *Rhizobium loti*, D12791; *Rhodobacter capsulatus*, D16427; *Rhodophila globiformis*,

D86513; *Rhodopseudomonas palustris*, D84187; *Rhodospirillum salinarum*, D14432; *Roseobacter litoralis*, X78312; *Roseobacter denitrificans*, M96746; *Roseococcus thiosulfatophilus*, X72908; *Sphingomonas adhaesiva*, D13722; *Sphingomonas yanoikuyae*, D13728; and strains RE35F/1 (AF118020) and SI85-9A1 (U53824).

RESULTS AND DISCUSSION

A yellow-pigmented Mn(II)-oxidizing bacterium, designated strain SD-21, was isolated from surface sediments of San Diego Bay, CA. When grown on Mn(II)-containing plates for over one week colonies become dark brown, due to the formation of Mn oxides on the cell surface. A phenotypically similar Mn(II)-oxidizing bacterium, strain MB-16, was also isolated from sediments of Mission Bay (San Diego, CA). Comparison of the 16S rRNA sequences of SD-21 and MB-16 revealed that these organisms were very closely related, sharing 99.7% identity over 1440 bases (differing by only 4 bases). Because of the similarity between these organisms, only one of the strains, SD-21, was fully characterized in this study. Database searches (BLAST and RDP) demonstrated that the 16S rRNA sequence of SD-21 was most closely related to that of "Erythrobacter citreus" strain RE35F/1 (98.9% identity, 1403 bases considered), a yellow-pigmented organism recently isolated from the 0.2 μm

filterable fraction of water samples obtained from the Mediterranean Sea (Vybiral et al., 1999). The 16S rRNA sequence of SD-21 was 97.4% and 97.3% identical (over 1403 bases) to the sequences of the type strains *Erythrobacter litoralis* and *Erythrobacter longus*, respectively. The next most closely related sequences (1402 bases considered) were from *Porphyrobacter neustonensis* (96.2%), *Erythromicrobium ramosum* (96.0%), and *Citromicrobium bathyomarinum* (95.5%), indicating that small differences (<1%) at the 16S rRNA level may correspond to genus-level physiological differences in this group of aerobic anoxygenic phototrophic bacteria. A phylogenetic tree (Fig. 1) based on closely related sequences in the databases and diverse representatives of the α-*Proteobacteria* indicates that strain SD-21 clusters with other known *Erythrobacter* species, within the alpha-4-subgroup of the *Proteobacteria*, forming an additional clade with RE35F/1. From this tree, it is clear that SD-21 is not closely related to other known Mn(II)-oxidizing α-proteobacteria (e.g., SI85-9A1, *Pedomicrobium manganicum*, etc.), providing further evidence that Mn(II) oxidation is not confined to a single group or lineage within the α-*Proteobacteria*.

Microscopic examination of SD-21 cultures revealed motile, Gram-negative, rods that were quite small, approximately 0.2 μm by 0.9 μm. Growth occurred over a wide range of conditions, including temperature (12° to 37°C), pH (6 to 9), and NaCl

concentration (1 to 8%). Optimal growth occurred at 25-30°C, pH 6.5-7.5, and 1.5-3.5% NaCl, respectively. SD-21 did not grow in freshwater K media, with or without added NaCl. This organism required NaCl as well as other artificial seawater constituents for growth, indicating that like *Erythrobacter* species it appears to be a true "marine" bacterium.

One of the defining and rather striking characteristics of *Erythrobacter* species is their dark orange-red ("erythrus" = red) pigmentation, which is due to the presence of extremely high amounts of carotenoids (Shiba and Simidu, 1982; Yurkov et al., 1994). The yellow pigmentation of strain SD-21, however, differed greatly from the more reddish pigmentation of both *E. longus* and *E. litoralis* when grown under the same conditions. Another defining characteristic of *Erythrobacter* species, and all aerobic anoxygenic phototrophic bacteria, is the presence of bacteriochlorophyll-*a* (Bchl-*a*) (Yurkov and Beatty, 1998), an essential component of the light-harvesting complexes of these organisms. To determine whether Bchl-*a* and carotenoids were present in SD-21, methanol/acetone extracts were obtained from dark-grown cells of SD-21 as well as *E. longus*, *E. litoralis*, and a yellow-pigmented *Sphingomonas* isolate, as reference strains. The extracts of both *Erythrobacter* species were dark orange, while those of SD-21 and the *Sphingomonas* isolate were pale yellow. Absorption spectra revealed not only that SD-21 has considerably lower amounts of

carotenoids than the *Erythrobacter* species, but that Bchl-*a* is undetectable in this organism. These findings are particularly interesting in light of the fact that Bchl-*a* was recently shown to be absent in the closest known relative of SD-21, the yellow-pigmented strain, RE35F/1 (Vybiral et al., 1999). In fact, that organism was described as being chemotaxonomically more similar to the genus *Sphingomonas* than to *Erythrobacter* (Vybiral et al., 1999), despite its close phylogenetic affiliation with the genus *Erythrobacter*. Finally, unlike SD-21, neither *E. longus* nor *E. litoralis* oxidized Mn(II) when grown on liquid or solid K media containing 100μM $MnCl_2$. Overall, the significant phylogenetic and phenotypic differences between SD-21 and the two established *Erythrobacter* type strains suggest that SD-21 (and RE35F/1) may represent a new species or possibly genus of bacteria.

Regardless of the precise taxonomic placement of SD-21, this organism is without question the first Mn(II)-oxidizer described within the alpha-4 subgroup of the *Proteobacteria*. The formation of visible brown Mn oxides in SD-21 cultures generally occurred just prior to the onset of stationary phase, after 3 to 4 days of growth in K media (Fig. 2). Although growth was essentially identical in Mn(II)-containing cultures incubated in light or darkness, the rate of Mn(II) oxidation appeared to be slower in the presence of light. This is interesting in that both photoinhibition of bacterial Mn(II) oxidation and photoreductive dissolution of Mn

oxides and have been reported to occur within near-surface waters of the ocean (Sunda and Huntsman, 1988; 1994). Cultures grown in the presence of Mn(II) reached higher overall cell densities than those grown without Mn(II), suggesting that either Mn(II) itself or Mn(II) oxidation somehow enhances the overall growth yield of SD-21. Although Mn(II) serves as a cofactor for many cellular enzymes, for most organisms Mn(II) is generally only required in trace amounts and, thus, would not be expected to limit bacterial growth. It is also unlikely, however, that SD-21 is capable of coupling growth to Mn(II) oxidation, since there have been no conclusive reports of this phenomenon in the literature. One possibility is that the Mn oxides that encrust the cells may enhance the overall growth yield by providing protection against various harmful agents (e.g., toxic oxygen species, proteases, metabolic byproducts, etc.) which may accumulate in the culture media over time. In the natural environment, the Mn oxides which encrust Mn(II)-oxidizing organisms have been proposed to serve a number of different biological functions, including: protection against ultraviolet light, toxic metals, viral attack, predation, etc.; oxidants of refractory organic matter into utilizable substrates; scavengers of trace metals required for growth; and storage of an electron acceptor for anaerobic respiration (Tebo et al., 1997). However, none of these are likely to explain the different growth yields of SD-21 cultures grown in the presence and absence of Mn(II).

SDS-PAGE analysis of protein extracts from cells collected throughout a 7-day time course revealed that active Mn(II)-oxidizing factors of ~250-kDa and 150-kDa were detectable after 60 h of growth (Fig. 3). This timing of expression corresponds to mid-logarithmic growth phase, which is just prior to the onset of detectable Mn(II) oxidation in cultures, as well as the point after which growth begins to deviate between cultures grown in the presence or absence of Mn(II). Thus, there may be a link between Mn(II) oxidation and the observed differences in growth. In *Pseudomonas putida* strains MnB1 and GB-1, Mn(II) oxidation is believed to be induced by starvation (De Pamla, 1993) and/or the onset of stationary phase (Jung and Schweisfurth, 1979; Okazaki et al., 1997). In SD-21, the onset of Mn(II) oxidation in cultures also varied depending on the type of peptone (e.g., trypticase, casamino acids, proteose, etc.) used in the media (data not shown), possibly indicating that Mn(II)-oxidation (expression of the Mn(II) oxidase) is influenced by the relative concentrations of specific amino acids or other trace contaminants present in the different peptones. The specific factors involved in the regulation of Mn(II) oxidation in SD-21 require further investigation.

The activity of the Mn(II)-oxidizing factors produced by strain SD-21 are extremely stable, capable of withstanding exposure to a variety of harsh conditions, including multiple freeze-thaw cycles, high concentrations of SDS (1%), and SDS-

PAGE under denaturing conditions (but not boiling). However, several lines of evidence suggest that these high-molecular-weight Mn(II)-oxidizing factors are actually multi-protein complexes. First, exposure of cell-free extracts to temperatures above 45°C results in the disappearance of both the Mn(II)-oxidizing bands and the corresponding Coomassie bands in gels (Fig.3, lane 160*), with the concomitant appearance of several distinct Coomassie-bands of ~100-kDa and ~140-kDa. This suggests heat-inactivation of the enzyme as well as the dissociation of the Mn(II)-oxidizing complexes into smaller protein components which lack detectable activity. Secondly, Mn(II)-oxidizing bands (as small as 50 to 60 kDa) have occasionally been observed to have activity in gels (data not shown). Thus, a single smaller protein is likely to be responsible for directly catalyzing the oxidation of Mn(II) but it may need to be present in multimeric form or in association with other proteins (and possibly cofactors) for optimal activity.

The involvement of multi-protein complexes in Mn(II) oxidation has also been reported in the model systems of *P. putida* GB-1 and *Bacillus* sp. strain SG-1. In *P. putida* GB-1, Mn(II)-oxidizing complexes with estimated molecular weights of 180 kDa and 250 kDa (Okazaki et al., 1997) were identified in native polyacrylamide gradient gels. These complexes are very similar in size to the Mn(II)-oxidizing factors found in strain SD-21. Although the product of the multicopper oxidase gene, *cumA*,

is believed to be a key component of the *P. putida* Mn(II)-oxidizing complexes (Brouwers et al., 1999; de Vrind et al., 1998), no activity has been observed outside of the complexes in a single protein of the appropriate size (~50 kDa). In spores of the marine *Bacillus* sp. strain SG-1, Mn(II)-oxidizing activity has only been consistently recovered in the form of a high-molecular-weight complex which barely enters low percentage SDS-polyacrylamide gels. As in *P. putida* GB-1, a multicopper oxidase, MnxG, appears to be the key protein involved in Mn(II) oxidation by SG-1 spores (van Waasbergen et al., 1996), but apparently requires the direct association with other proteins for activity.

The results of inhibitor assays revealed additional parallels with the model Mn(II)-oxidizing organisms and mulicopper oxidases. The Mn(II)-oxidizing activity of SD-21 cell-free extracts was inhibited at azide concentrations greater than 1 mM. This is significant because azide strongly inhibits multicopper oxidases, by bridging the type 2 and type 3 copper sites (Solomon et al., 1996), and also inhibits Mn(II) oxidation by *P. putida* GB-1, *L. discophora* SS-1, and the *Bacillus* sp. strain SG-1 (Boogerd and deVrind, 1987; Okazaki et al., 1997; Rosson and Nealson, 1982). In addition, the in-gel Mn(II)-oxidizing activity of SD-21 was completely inhibited by the copper chelator, o-phenanthroline (data not shown), at a concentration (50μM) well below the Mn(II) concentration (200μM). These results are similar to previous

findings in *P. putida* GB-1 and *Pedomicrobium* sp. ACM 3067 (Larsen et al., 1999; Okazaki et al., 1997) in which Mn(II) oxidation was also inhibited by the copper chelators, *o*-phenanthroline and DDC respectively, consistent with the involvement of multicopper oxidases.

Finally, like all known multicopper oxidases (e.g., laccase, ceruloplasmin, ascorbate oxdidase, etc.) (Solomon et al., 1996), the Mn(II)-oxidizing proteins of SD-21 are also capable of directly oxidizing various organic compounds, including p-phenylenediamine, in gels (Fig. 4). This is particularly analogous to the Fe(II)-oxidizing multicopper oxidase, FET3, from yeast which is capable of oxidizing both Fe(II) and p-phenylenediamine, but has a much higher affinity (450-fold) for Fe(II) than the organic compound (de Silva et al., 1997). Although the Mn(II)-oxidizing protein of SD-21 clearly has a number of properties in common with multicopper oxidases, definitive proof of this awaits the purification and characterization of large quantities of active enzyme, as well as isolation and sequencing of the responsible underlying gene. This protein is particularly well suited for both biochemical and spectroscopic studies, due to its stability over a wide range of conditions. Overall, SD-21 may serve as a useful model Mn(II)-oxidizing bacterium for not only studying the mechanism of Mn(II) oxidation within the α-*Proteobacteria*, but also the biological function of bacterial Mn(II) oxidation.

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Figure 1. Neighbor-joining phylogenetic tree showing the relationship of strain SD-21 to closely related members of the alpha-4-subgroup of the *Proteobacteria* as well as diverse representatives of the α-*Proteoacteria*. Percentages of bootstrap support (>60%) from 1,000 replicates are indicated at the branch points.

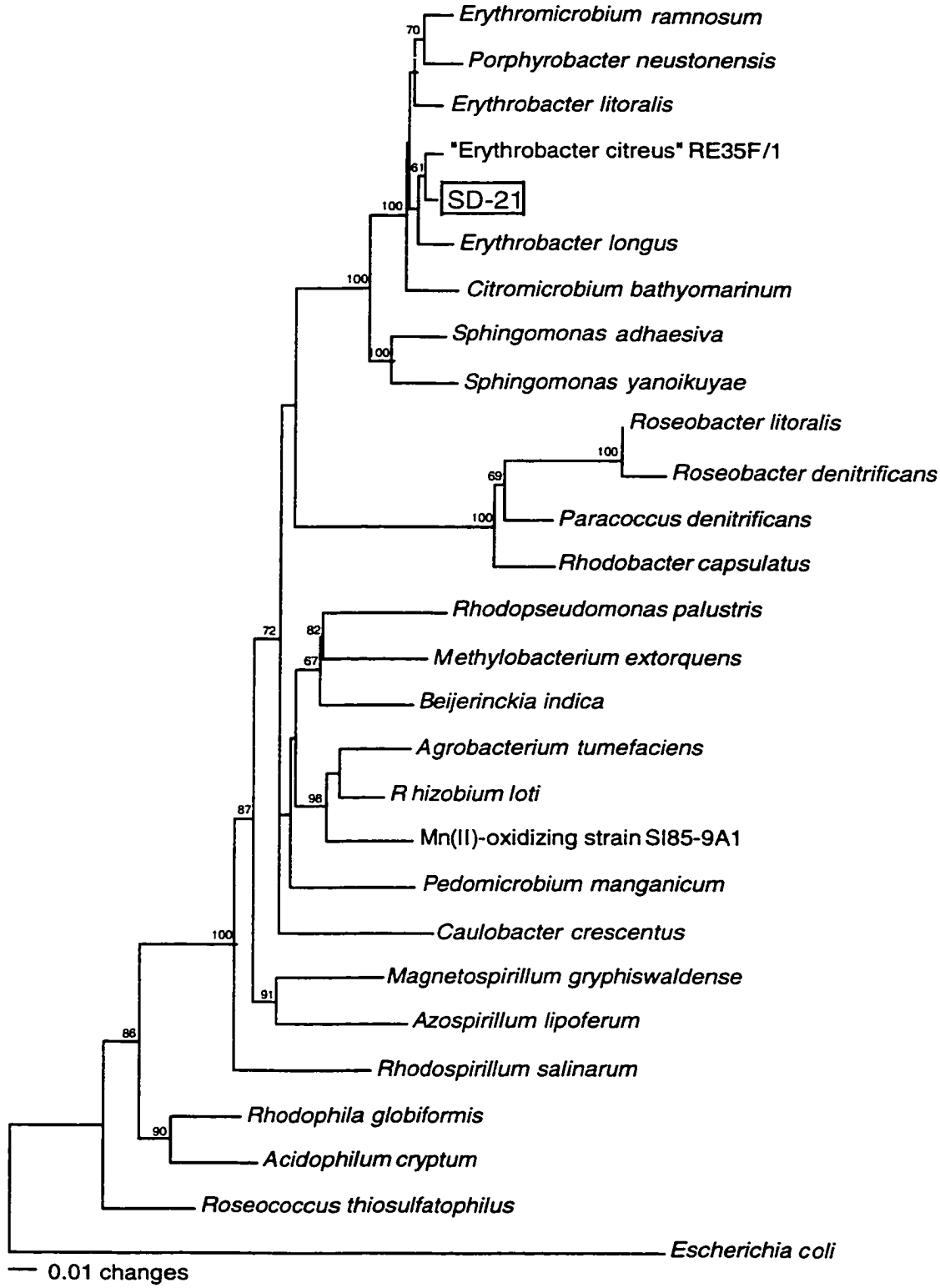


Figure 2. Growth and Mn(II) oxidation by SD-21 under different light and metal regimes. Filled symbols correspond to dark-grown cultures and empty symbols correspond to light-grown cultures. Triangles correspond to cultures grown in the presence of 100μM $MnCl_2$, while circles correspond to cultures grown without added Mn(II). The production of Mn oxides in cultures was measured using leucoberbelin blue.

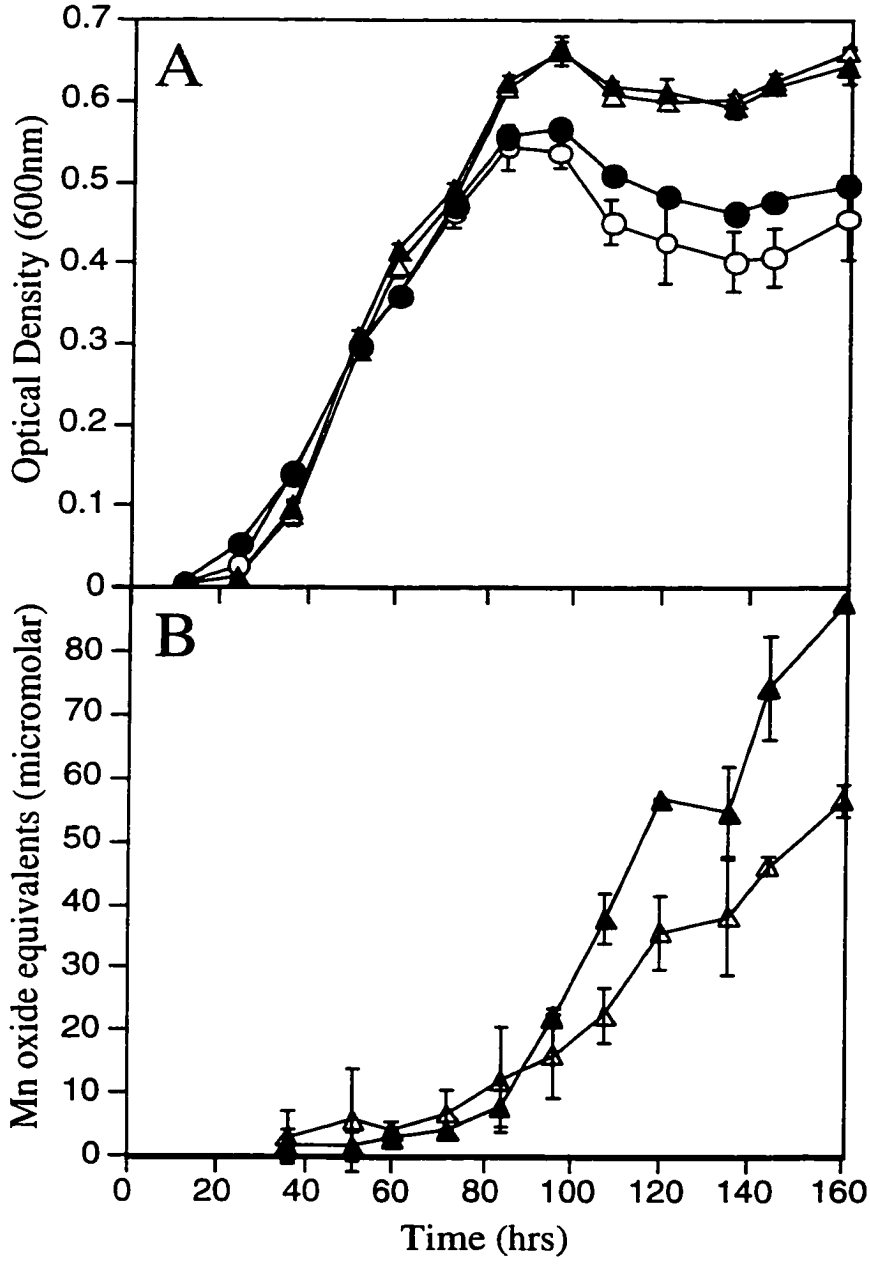


Figure 3. Expression of the Mn(II)-oxidizing protein(s) during the time-course shown in Figure 2. Duplicate gels of cell-free extracts collected from time points throughout the growth curve were stained for total protein (left) or assayed for in-gel Mn(II)-oxidizing activity (right) by incubation in a Mn(II)-containing buffer. Arrows correspond to the Coomassie-stained proteins responsible for Mn(II) oxidation. The last sample in both gels (160*) was heat-treated at 65°C for 5 minutes prior to electrophoresis.

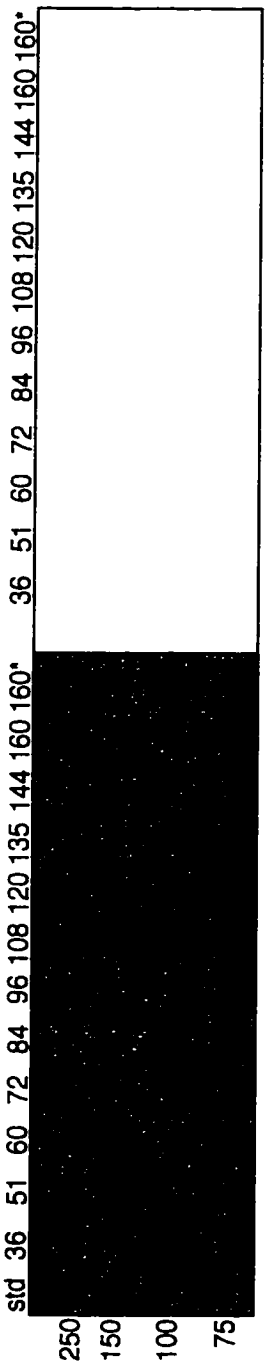
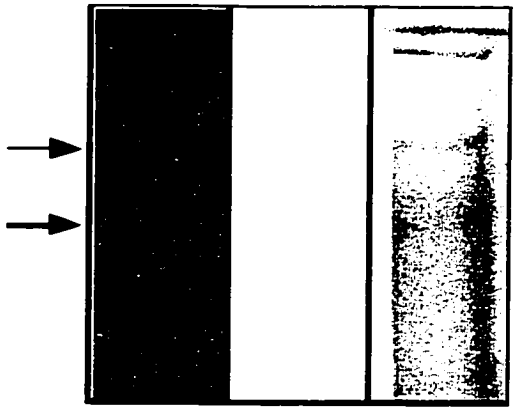


Figure 4. In-gel oxidation of both Mn(II) and the organic substrate, p-phenylenediamine (pPD), by the 150 kDa and 250-kDa proteins of SD-21. Gels were incubated in either Coomassie blue (lane 1), Mn(II) buffer (lane 2), or a HEPES-buffered solution of pPD (lane 3).



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CHAPTER VII

Conclusions and Future Directions

CONCLUSIONS AND FUTURE DIRECTIONS

The key role of bacteria in catalyzing Mn(II) oxidation has been recognized for over a century, but the underlying mechanisms are still not well understood today. In recent years, molecular genetic approaches have revealed the involvement of multicopper oxidases in Mn(II) oxidation in three phylogenetically distinct bacteria: the marine *Bacillus* sp. strain SG-1, *Leptothrix discophora* SS-1, and *Pseudomonas putida* GB-1. Although these organisms have served as useful model systems, representing genera ubiquitous in many different environments, prior to my research, virtually nothing was known regarding the diversity of organisms capable of oxidizing Mn(II) within these genera. In addition, it was not known whether the Mn(II) oxidation-associated multicopper oxidase genes were confined to closely related organisms or whether they might instead occur in diverse species throughout these genera, including non-oxidizing strains. This dissertation has addressed a number of these issues by using a molecular approach to explore both the diversity and molecular mechanisms of Mn(II) oxidation within the environmentally-important genera, *Bacillus* and *Pseudomonas*. In addition, the mechanism of Mn(II) oxidation was investigated within a new marine Mn(II)-oxidizing α-proteobacterium, which has a number of features that make it an attractive model system.

In **Chapter 3**, the Mn(II)-oxidizing activity and the putative Mn(II) oxidase, MnxG, were definitively localized to the outermost layer of spores of the marine *Bacillus* sp. strain SG-1. Prior to this study, genetic studies indicated that the product of the *mnxG* multicopper oxidase gene was a key factor involved in Mn(II) oxidation in SG-1, but this protein had not yet been definitively localized to the spore. To address this, MnxG-specific antisera were generated and used to probe the outermost layer of SG-1 spores. This task was greatly facilitated by utilizing the French press to effectively remove the Mn(II)-oxidizing outer layer from SG-1 spores, without disrupting activity. This layer appears to be ultrastructurally and chemically distinct from the spore coat, possibly representing an exosporium. Because so little is known regarding the proteins, activities, or possible functions associated with exosporia, the results of this study may make a significant contribution to this area of spore physiology. To my knowledge, Mn(II) oxidation is the first oxidase activity, and MnxG one of the first gene products, ever shown to be associated with such an outermost spore layer. In the future, it would be informative to determine which of the other *mnx* gene products might also be associated with the outermost layer. This could be achieved through the development of antisera to other *mnx* gene products or possibly by careful analysis of protein banding patterns of outer layer extracts from various mutants within the *mnx* gene cluster. It will also be important to examine the

composition of inorganic constituents within the outermost layer (e.g., phosphate, metals, calcium, etc.), which would not be detected through standard analyses of protein, lipid, and carbohydrate. Ultrastructural and chemical analysis of outer layers from diverse Mn(II)-oxidizing spore-formers might reveal whether there is a consistent link between spore physiology and Mn(II) oxidation.

Despite years of intensive studies of the genes and proteins involved in the formation, germination, and resistance of bacterial spores, there have been no other reports of spore-associated activities similar to Mn(II) oxidation by SG-1 spores. For this reason, in **Chapter 4** I investigated how widespread this phenomenon was within the genus *Bacillus* by analyzing a number of Mn(II)-oxidizing spore-formers isolated from coastal marine sediments. This revealed that organisms capable of producing Mn(II)-oxdizing spores are phylogenetically diverse within the genus *Bacillus*. However, it was also established that Mn(II) oxidation is not a universal trait within the genus *Bacillus*, since a variety of well-known *Bacillus* species did not produce Mn(II)-oxidizing spores. The active Mn(II)-oxidizing proteins recovered from all of the Mn(II)-oxidizing isolates in SDS-PAGE gels represent the first Mn(II)-oxidizing enzymes ever identified in spores or marine bacteria. The sensitivity of these enzymes to both azide and the copper-chelator, o-phenanthroline, suggests the direct involvement of metalloenzymes (e.g., multicopper oxidases) in Mn(II) oxidation. This

was further supported by the fact that highly conserved *mnxG* sequences were obtained from all of the isolates, suggesting the existence of a common Cu-dependent mechanism for Mn(II) oxidation in phylogeneticllay diverse *Bacillus* strains. In light of these studies, the commonly held view that spores are totally inactive structures in the environment should be revised. In the future, it will be essential to obtain large amounts of active Mn(II)-oxidizing protein(s) for detailed biochemical and spectroscopic analysis. Peptide sequencing of these proteins would confirm whether these enzymes are, in fact, multicopper oxidases (e.g., MnxG). The underlying genes could then be cloned, sequenced, and overexpressed heterologously (in bacteria or yeast). If activity can be recovered from the expressed proteins, this would open the door for a wide range of studies, including site-directed mutagenesis to determine the specific amino acid residues that are essential for binding and oxidizing Mn(II). Since MnxG appears to be involved in phylogenetically diverse Mn(II)-oxidizing spore formers, it might be useful to generate specific peptide-antibodies to regions of the protein conserved in all of the known sequences. These antibodies could be used to definitively determine whether the Mn(II)-oxidizing enzymes in SDS-PAGE gels are MnxG and also to detect MnxG within the high-molecular-weight protein complexes associated with SG-1 spores. Finally, it will be important to continue isolating and characterizing Mn(II)-oxidizing spore-formers from diverse environments (e.g., deep-

sea sediments, hydrothermal vents, Mn nodules, hypersaline environments, etc.), in order to more fully assess how important these metabolically-dormant catalysts are in nature and within the genus *Bacillus*.

Although many *Pseudomonas* isolates have been reported to oxidize Mn(II), only the *P. putida* strains GB-1 and MnB1 have been well-characterized. In **Chapter 5**, I demonstrated that the capacity to oxidize Mn(II) occurs in phylogenetically diverse *Pseudomonas* strains. In addition, the Mn(II)-oxidation-associated multicopper oxidase gene, *cumA*, was found to be present in both Mn(II)-oxidizing and non-oxidizing strains. This suggests that the *cumA* gene may either be functionally inactive (e.g., not expressed) or have an alternative function in non-oxidizing strains. Many of the Mn(II)-oxidizing strains were also found to be capable of oxidizing the synthetic organic laccase-substrate, ABTS. The inability of non-oxidizing (*ccm* and *cumA*) mutants of *P. putida* GB-1 and MnB1 to oxidize ABTS suggests a direct link between the Mn(II)-oxidizing system and the oxidation of organic substrates. This also raises the possibility that bacterial "Mn(II) oxidases" may be multi-functional oxidases capable of oxidzing a variety of substrates. This should not come as a surprise, since all known multicopper oxidases are capable of oxidizing organics to some extent. However, to address this issue, it will be necessary to purify large quantities of active protein from these pseudomonads, so that the

relative affinities for metal and organic substrates can be determined. In addition to model synthetic compounds, like ABTS, various "natural" laccase substrates (e.g., syringaldazine, guaicol, diphenolics) should be tested as well. To address the issue of whether *cumA* is actually expressed in non-oxidizing strains, techniques such as Northern analysis or reverse-transcriptase PCR (RT-PCR) should be used. It would also be useful to generate specific antibodies to conserved regions of CumA. These antisera could be used to analyze CumA expression in both Mn(II)-oxidizing and non-oxidizing strains, as well as to localize the protein within the high-molecular-weight Mn(II)-oxidizing complexes. If peptide antibodies were generated to highly conserved copper-binding regions (e.g., HPIHLHGM) found in a variety of multicopper oxidases, these antisera could be used to look for the presence of multicopper oxidases in phylogenetically diverse Mn(II)-oxidizing bacteria (e.g., α-proteobacteria). Finally, the *cumA* gene must be evaluated as a functional gene probe for the detection of Mn(II)-oxidizing organisms in the natural environment. It will be particularly important to not only detect the presence of the *cumA* gene, but also the transcript, in nucleic acid extracts obtained from Mn(II)-oxidizing microenvironments (e.g., oxic-anoxic transition zones, ferromanganese particles, etc.).

Although α-*Proteobacteria* are extremely abundant within both the culturable and unculturable microbial populations in the ocean, the process of Mn(II) oxidation

has not been investigated to a significant extent within these organisms. In **Chapter 6**, I investigated the biochemical basis for Mn(II) oxidation within a new Mn(II)-oxidizing α-proteobacterium, strain SD-21, obtained from San Diego Bay sediments. The fact that a virtually identical strain, MB-16, was isolated from from Mission Bay sediments suggests that these organisms may be prevalent in coastal marine sediments. In addition, these strains are phylogenetically most closely related (~99% at the 16S rRNA level) to an organism obtained from the water column of the Mediterranean Sea, suggesting that these organisms may be present in a variety of marine environments. SD-21 is the first Mn(II)-oxidizer reported within the α-4-subgroup of the *Proteobacteria*. Although related to the orange-pigmented, bacteriochlorophyll-a-containing genus *Erythrobacter*, SD-21 has a number of distinguishing features, suggesting that it may be a new species or genus. In SD-21 cultures, Mn(II) oxidation becomes detectable during early stationary phase of growth, yet the Mn(II)-oxidizing proteins are detectable in SDS-PAGE gels during logarithmic phase. The Mn(II)-oxidizing factors (~250-kDa and 150-kDa) of SD-21 are sensitive to azide and *o*-phenanthroline, suggesting the possible involvement of a multicopper oxidase-like enzyme. The stability and relative abundance of these Mn(II)-oxidizing proteins makes them attractive candidates for future biochemical and spectroscopic studies. Since this is the first example of a Mn(II)-oxidizing protein directly oxidizing an

organic substrate, future studies should focus on determining the relative affinities of these enzymes for metal vs. organic substrates. In addition, it should be possible to obtain peptide sequence from the Coomassie-stained bands in gels, which would allow for the eventual cloning, sequencing, and overexpression of the gene(s) involved in Mn(II) oxidation. Future physiological studies should also focus on understanding how Mn(II) oxidation is reduced or inhibited by light as well as how growth is enhanced by the presence of Mn(II). Overall, these studies should provide important clues regarding the potential mechansims and functions of bacterial Mn(II) oxidation in the environment.