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Microbial Manganese(II) Oxidation: Biogeochemistry of a Deep-Sea Hydrothermal Plume, Enzymatic Mechanism, and Genomic Perspectives

A Dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Marine Biology

by

Gregory J. Dick

Committee in charge:

Bradley M. Tebo, Chair
Farooq Azam
Douglass H. Bartlett
Katherine A. Barbeau
Seth M. Cohen
Kit J. Pogliano

2006

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Chair

University of California, San Diego

2006

This dissertation is dedicated to my family:

My parents, Steve and Terry, my brother, Anthony, and my wife, Jenna

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Dave and Jay helped me explore some of the more beneficial applications of microbiology.

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Tebo, B.M., B.G. Clement, and **G.J. Dick**. Microbial transformations of Manganese. In: Manual of Environmental Microbiology, ASM Press, in review.

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Biological Oceanography
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Marine Biochemistry
Professors Douglas Bartlett, Horst Felbeck, and Victor Vacquier

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ABSTRACT OF THE DISSERTATION

Microbial Manganese(II) Oxidation: Biogeochemistry of a Deep-Sea Hydrothermal Plume, Enzymatic Mechanism, and Genomic Perspectives

by

Gregory J. Dick

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2006

Bradley M. Tebo, Chair

Manganese (Mn) oxides are highly reactive minerals that play an important role in elemental biogeochemical cycles, controlling the speciation and availability of many metals and organic compounds. Microbes catalyze the transformation of soluble Mn(II) into solid-phase Mn(III/IV) oxides, however the identity of the organisms responsible and mechanism of this biomineralization are unknown. Field work was carried out in deep-sea hydrothermal vent plumes at Guaymas Basin (GB) in the Gulf of California, where previous studies suggested that microbes play a major role in the oxidation precipitation of Mn. Mn(II) oxidation rates were measured using ^{54}Mn as a radioactive tracer and were found to be the fastest ever recorded in a deep-sea hydrothermal plume. This rapid

Mn(II) oxidation is catalyzed by microbial enzymes and is specific to the GB plume, suggesting that a distinct community of microbes is present in the plume compared to background deep-seawater. However, molecular methods revealed that there are only subtle differences in the microbial community in the plume versus background deep-seawater, indicating that the unique biogeochemistry of the plume is due to unique activities of plume microbes rather than unique types of plume microbes. Mn(II)-oxidizing isolates were not well represented in plume clone libraries, suggesting that the organisms responsible for Mn(II) oxidation in the plume have not yet been identified. Laboratory investigations of model Mn(II)-oxidizers in the lab further elucidated the mechanism of Mn(II) oxidation. The Mn-oxidizing enzyme from spores of a marine *Bacillus* species was partially purified and identified by tandem mass spectrometry (MS/MS), providing a conclusive match to the protein MnxG, a multicopper oxidase. These results demonstrate that MnxG directly catalyzes the oxidation of Mn(II) to Mn(IV) oxides, a novel biochemical reaction for a multicopper oxidase. Genome sequence analaysis of the Mn(II)-oxidizing α-proteobacterium strain SI85-9A1 provided genomic insights into Mn(II) oxidation. The genome encodes metabolic versatility, including pathways for heterotrophy, lithotrophy (on sulfur, methanol, CO), and authotrophy (via the Calvin cycle). Genes encoding the putative Mn(II) oxidase were identified and found to be widespread in completed proteobacterial genomes and in environmental datasets, suggesting that Mn(II) oxidation is more widespread that previously recognized.

I

Introduction

In recent years, it has become increasingly clear that microbes drive a number of geochemical processes previously thought to be abiotic, including the formation and dissolution of minerals. Perhaps more surprising is the level at which microbes are involved in these processes. For example, whereas microbes have long been recognized as reactive surfaces that can influence the distribution and availability of elements in nature, it is now clear that many biogeochemical transformations are driven by enzymes whose activity is inextricably linked to the identity, genetics, and physiology of the organisms involved. Manganese (Mn) is a perfect example of an element whose cycling is driven by the activities of microorganisms; this dissertation focuses on microbial catalysis of the oxidative segment of the Mn cycle. Biogeochemical data suggests that microbes mediate Mn(II) oxidation in many environments where Mn cycling is prevalent, and culture-based studies suggest that microbes can oxidize Mn(II) enzymatically. However, major gaps in understanding microbial Mn(II) oxidation include knowledge of what microbes are environmentally relevant, what the biochemical mechanisms are, and what the physiological benefit of Mn(II) oxidation is. Here I review what is known about Mn biogeochemistry, the microorganisms involved, and the genetic and biochemical mechanisms that underpin it. I then introduce the data chapters that follow, and summarize how they relate to each other.

Mn biogeochemistry

Mn can exist as several oxidation states ranging from 0 to +7, however the three oxidation states that predominate under environmental conditions are +2, +3, and +4 (for a review of the chemistry discussed here, see (Tebo et al., 2004). In general, Mn(II)

occurs as a cation in solution and is thermodynamically favorable under anoxic or reducing conditions. Mn(IV) occurs as solid-phase Mn oxides or hydroxide minerals and is the thermodynamically favored form under oxic conditions at circumneutral pH. Mn(III) is thermodynamically unstable and disproportionates to Mn(II) and Mn(IV) in the absence of organic compounds that chelate and stabilize Mn(III). For this reason Mn(III) was not thought to be abundant in nature, however recent data suggest that it be more abundant than previously recognized. First, Mn(III) is an intermediate in the biological oxidation of Mn(II) to Mn(IV) (Webb et al., 2005a). Second, Mn(II)-oxidizing organisms produce siderophores that bind and complex Mn(III) with high affinity, providing a possible sink for Mn(III) produced by Mn(II)-oxidizing bacteria (Parker et al., 2004). Third, Mn(III) has now been measured and is present in appreciable quantities in suboxic zones where Mn cycling is prevalent (Trouwborst et al., 2006). Transformations of Mn among its oxidation states are kinetically slow by chemical mechanisms alone, and microorganisms are thought to catalyze Mn oxidation rates that are observed in many environments (Cowen et al., 1986; Cowen et al., 1990; Fuller and Harvey, 2000; Harvey and Fuller, 1998; Kay et al., 2001; Mandernack and Tebo, 1993; Marble, 1998; Marble et al., 1999; Nealson et al., 1988; Tebo, 1991; Tebo and Emerson, 1985; Tebo and Emerson, 1986; Tebo et al., 1984; Tebo et al., 1985; Tipping, 1984; van Cappellen et al., 1998; Wehrli et al., 1995). For this reason, most naturally occurring Mn oxide minerals are thought to be the result of microbial activities (Tebo et al., 2004).

Microbial Mn(II) oxidation requires O_2 and produces H^+, and based on measurements of these reactants/products, it is typically described with the following chemical reaction:

$$Mn^{2+} + 1/2O_2 + H_2O \Rightarrow MnO_2 + 2H^+$$

The products of microbial Mn(II) oxidation are poorly crystalline, highly amorphous Mn oxides that resembles δ-MnO_2 or birnessite (Tebo et al., 2004). As strong scavengers, these biogenic Mn oxides control the distribution and availability of many trace elements, and as the strongest environmentally relevant oxidant after oxygen, they serve as electron acceptors for anaerobic respiration (Tebo et al., 2004). Because of this broad influence that Mn oxides exert on biogeochemical cycles, it is important to understand the biological mechanism of their formation.

Mn(II)-oxidizing Microorganisms and Mechanisms

Microorganisms that oxidize Mn(II) to Mn(III/IV) oxides are widespread in nature and include both bacteria and fungi. While Mn oxide biogenesis by fungi has only recently begun to be investigated (Miyata et al., 2006; Schlosser and Höfer, 2002), many phylogenetically diverse Mn(II)-oxidizing bacteria have been described. Based on 16S rRNA gene sequences, all belong to the low (Firmicutes) and high (Actinobacteria) GC Gram-positive and the α, β, and γ Proteobacteria branches of the Domain Bacteria. Despite the fact that diverse microbes have the ability to oxidize Mn(II), the physiological function of Mn(II) oxidation is unknown. In the final step of Mn(II) oxidation bacteria often become encrusted in manganese oxides, usually at the onset of stationary phase or after sporulation. Although the oxidation of soluble Mn(II) to Mn(III/IV) oxides is energetically favorable and some evidence suggests that microbes are able to harness and utilize the energy of this reaction (Ehrlich, 1983; Ehrlich and

Salerno, 1990; Kepkay and Nealson, 1987), definitive proof remains elusive (Brouwers et al., 2000b; Tebo et al., 1997b; Tebo et al., 2005). One Mn(II)-oxidizing bacterium has even been shown to possess the genes for ribulose-1,5-bisphosphate carboxylase/oxygenase (Caspi et al., 1996), the enzyme responsible for CO_2 fixation in most aerobic chemolithoautotrophs. However, no correlation between Mn(II) oxidation and CO_2 fixation could be demonstrated. Other biological functions of Mn(II) oxidation have been proposed, including protection from toxic heavy metals or reactive oxygen species (Ghiorse, 1984); UV light, predation, or viruses (Emerson, 1989); storage of an electron acceptor for later use in anaerobic respiration (Tebo, 1983); breakdown of refractory organic matter into utilizable substrates (Sunda and Kieber, 1994); and scavenging of micronutrient trace metals. These potential biological functions of microbial Mn(II) oxidation are discussed extensively elsewhere (Brouwers et al., 2000b; Tebo et al., 1997b; Tebo et al., 2005).

Bacteria catalyze the oxidation of Mn(II) by direct and indirect processes. Indirect catalysis of Mn(II) oxidation occurs when organisms (1) modify the pH and/or redox conditions of the local aqueous environment, or (2) release metabolic end products that chemically oxidize Mn(II) (Hullo et al., 2001; Richardson et al., 1988). Bacteria have long been known to oxidize Mn(II) directly via the production of polysaccharides (Beveridge, 1989; Ghiorse and Hirsch, 1979; van Veen et al., 1978) or proteins (Adams and Ghiorse, 1987b; Boogerd and de Vrind, 1987; de Vrind et al., 1986; Douka, 1980; Ehrlich, 1968; Ehrlich, 1983; Francis et al., 2001; Jung and Schweisfurth, 1979; Larsen et al., 1999; Okazaki et al., 1997) that catalyze the reaction , but only recently have the

biochemical and underlying genetic details begun to be elucidated. Three model Mn(II)-oxidizing organisms have been studied extensively using molecular biological techniques: *Leptothrix discophora* SS-1 (Brouwers et al., 2000a; Corstjens et al., 1997), *Pseudomonas putida* strains MnB1 and GB-1 (Brouwers et al., 1999; Brouwers et al., 1998; Caspi, 1996; Caspi et al., 1998; de Vrind et al., 2003; de Vrind et al., 1998), and *Bacillus* sp. strain SG-1 (Francis et al., 2002; Francis and Tebo, 2002; van Waasbergen et al., 1996; van Waasbergen et al., 1993). These three Mn(II)-oxidizers are phylogenetically distinct, representing the β-proteobacteria, γ-proteobacteria, and low-GC gram-positive bacteria, respectively. All three oxidize Mn(II) enzymatically on an exopolymer matrix surrounding the cell: *Leptothrix discophora* on an extracellular sheath (though strain SS-1 is a sheathless mutant that secretes the Mn(II)-oxidizing factor into the medium), MnB1/GB-1 on the outer membrane glycocalyx, and SG-1 on the exosporium (the outermost layer of the spore). Molecular genetic approaches have revealed an exciting connection: All three organisms possess genes that are involved in Mn(II) oxidation which share sequence similarity with multicopper oxidase enzymes. Biochemical evidence from these organisms and two α-proteobacteria, a *Pedomicrobium* species (Larsen et al., 1999) and the *Erythrobacter*-like strain SD-21 (Francis et al., 2001) is also consistent with the involvement of copper enzymes in Mn(II) oxidation. In all the organisms tested, copper ions appear to be required for Mn(II) oxidation (Brouwers et al., 2000a; Larsen et al., 1999; van Waasbergen et al., 1996). Multicopper oxidases, a diverse family of enzymes, utilize multiple types of copper centers as cofactors to oxidize a variety of organic and inorganic substrates (Solomon et al., 1996). Multicopper oxidases oxidize a wide range of substrates in one-electron steps and reduce

O_2 to water. Sequence similarity between the putative bacterial Mn(II) oxidases and more well-studied eukaryotic multicopper oxidases (ceruloplasmin in humans, ascorbate oxidase from plants, and laccase from plants and fungi) is limited to very specific sites within the proteins (the copper binding sites). Beyond what can be inferred from the limited sequence similarity, little is known about the putative bacterial Mn(II) oxidases.

The role of multicopper oxidases in bacterial Mn(II) oxidation

Despite recent advances in identifying proteins involved in bacterial manganese oxidation and in elucidating the mechanism by which it occurs, many central questions remain unanswered. The mere involvement of multicopper oxidases in Mn(II) oxidation must be distinguished from actual direct catalysis of Mn(II) oxidation by multicopper oxidases. Only in the case of *Leptothrix discophora* has a potential link been made between the Mn(II)-oxidase and an underlying multicopper oxidase gene (Corstjens et al., 1997). No bacterial Mn(II) oxidase has been purified in quantities sufficient for detailed biochemical study, and to date, no multicopper oxidase gene thought to encode a Mn(II) oxidase has been successfully expressed (i.e., active) in a foreign host. Thus the direct role of multicopper oxidases in Mn(II) oxidation remains a hypothesis. Nevertheless, because (1) genetic and biochemical studies point to the involvement of multicopper oxidases in Mn(II) oxidation in several unrelated bacteria, (2) some eukaryotic multicopper oxidases are known to directly oxidize Mn(II) (Höfer & Schlosser 1999, Schlosser & Höfer 2002(Miyata et al., 2006)) and (3) multicopper oxidases that oxidize Fe(II) occur in both eukaryotes (Solomon et al., 1996) and bacteria (Huston et al., 2002;

Kim et al., 2001), it seems reasonable that multicopper oxidases in bacteria could also directly oxidize Mn(II).

A general feature of multicopper oxidases is their broad substrate specificity: many are able to oxidize multiple substrates including both metals and organics (Solomon et al., 1996). A low substrate specificity may also be common for Mn(II) oxidases; Francis and Tebo (2001) found that only *Pseudomonas* strains capable of oxidizing Mn(II) could oxidize the organic compound ABTS. Future work is needed to further characterize the substrate specificity of bacterial Mn(II)-oxidases. Do Mn(II) oxidases feature a substrate-binding pocket that ensures greater specificity as in ascorbate oxidase and ceruloplasmin (Solomon et al., 1996), or are they non-specific like the plant and fungal laccases? Perhaps the answers to such questions will shed light on the functional role of bacterial Mn(II) oxidation.

The question of bacterial multicopper oxidase specificity and function has broadened recently as genome sequencing has revealed that multicopper oxidases, identified by their copper binding site motifs, are prevalent in bacteria (Alexandre and Zhulin, 2000; Claus, 2003). Although most of these putative bacterial multicopper oxidases are uncharacterized, results so far indicate that they are involved in a wide range of functions, including copper resistance (Kim et al., 2001; Lee et al., 1994; Mellano and Cooksey, 1988; Yang et al., 1996), melanin production/UV protection (Hullo et al., 2001; Solano et al., 2000), and iron oxidation and acquisition (Huston et al., 2002). One study showed that YacK, a multicopper oxidase from *E. coli*, exhibits oxidase activity towards both iron and organic compounds, and suggested functional roles for both activities (Kim

et al., 2001). It is interesting that this and another (Hullo et al., 2001) bacterial multicopper oxidase are unable to oxidize Mn(II). Genome sequencing of *Pseudomonas putida* KT2440, *P. fluorescens* strain PfO-1, and *Nitrosomonas europaea* has revealed that these organisms contain genes similar to *Bacillus* sp. *mnxG*, while KT2440 also has a putative *cumA* gene. The putative proteins encoded by these three *mnxG*-like genes are from distantly related bacteria and share a high degree of sequence similarity, suggesting perhaps that they represent a distinct class of multicopper oxidases.

Organization of the dissertation

The work I present in this dissertation was motivated by questions that have inspired investigations of microbial Mn(II) oxidation for decades: What microorganisms are responsible? What genes/enzymes are involved and what are the biochemical mechanisms? Why do organisms oxidize Mn(II)? The dissertation involves two primary approaches to address these questions, field-based studies in a natural system, and laboratory-based studies on model organisms. The natural system is Guaymas Basin (GB) in the Gulf of California, a setting that hosts deep-sea hydrothermal activity where Mn(II) oxidation is extremely rapid. In **Chapter II**, Mn(II)-oxidizing *Bacillus* spores isolated from GB are described and characterized. In **Chapter III**, Mn(II) oxidation rates in the GB plume are measured and found to rapid, enzymatically catalyzed, and specific to the plume. In **Chapter IV**, molecular methods are used to analyze the microbial diversity of the GB hydrothermal plume, and to compare it to background Gulf of California deep-seawater. Chapters 5 and 6 describe laboratory investigations of model Mn(II)-oxidizing microbes in culture; in **Chapter V**, I report the first direct identification

of a Mn(II)-oxidizing enzyme, and in **Chapter VI**, I describe genomic insights into Mn(II) oxidation by a globally distributed α-proteobacterium.

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II

Manganese(II)-oxidizing *Bacillus* spores in Guaymas Basin hydrothermal sediments and plumes

Abstract

Microbial oxidation and precipitation of manganese at deep-sea hydrothermal vents is an important oceanic biogeochemical process, yet nothing is known about the types of microorganisms or mechanisms involved. Here we report the isolation of a number of diverse spore-forming Mn(II)-oxidizing *Bacillus* species from Guaymas Basin, a deep-sea hydrothermal vent environment in the Gulf of California, where rapid microbially-mediated Mn(II) oxidation was previously observed. *mnxG* multicopper oxidase genes involved in Mn(II) oxidation were amplified from all Mn(II)-oxidizing *Bacillus* spores isolated, suggesting that a copper-mediated mechanism of Mn(II) oxidation could be important at deep-sea hydrothermal vents. Phylogenetic analysis of 16S rRNA and *mnxG* genes revealed that while many of the deep-sea Mn(II) oxidizing *Bacillus* species are very closely related to previously recognized isolates from coastal sediments, others represent novel strains and clusters. Growth and Mn(II) oxidation properties of these *Bacillus* species suggest that in hydrothermal sediments they are likely present as spores that are active in oxidizing Mn(II) as it emerges from the seafloor.

Introduction

Deep-sea hydrothermal vents are a major source of dissolved Mn(II) for the world's oceans (Edmond et al., 1982), and Mn has long been used as a valuable tracer of hydrothermal vent emissions (Bolger et al., 1978; Coale et al., 1991; Edmonds et al., 2003; Klinkhammer et al., 1985; Klinkhammer and Hudson, 1986; Massoth et al., 1998). Hydrothermal fluids mix with oxygenated seawater in hydrothermal plumes, where

dissolved Mn(II) is oxidized and precipitated by microorganisms to form particulate Mn(III/IV) oxides. These biogenic minerals are biogeochemically important because they can scavenge and/or oxidize a number of elements and compounds (Tebo et al., 2004), and because they contribute to metalliferous sediments and deposits that are widespread in areas surrounding mid-ocean ridges and back arc basins (Edmond et al., 1982; Klinkhammer and Hudson, 1986). Although bacteria are known to catalyze Mn(II) oxidation in many environments (Tebo et al., 2004), including deep-sea hydrothermal vents (Campbell et al., 1988; Cowen et al., 1998; Cowen and Li, 1991; Cowen et al., 1986; Cowen et al., 1990; Mandernack and Tebo, 1993), very little is known regarding the organisms or mechanisms involved in this oxidation/precipitation process.

Guaymas Basin (GB) in the Gulf of California (Fig. 2.1) is a hydrothermally-impacted, semi-enclosed basin, where Mn(II) oxidation and precipitation of Mn oxides is particularly intense (Campbell et al., 1988). It is an unusual hydrothermal system due to its close proximity to the coast, where high sedimentation rates maintain a thick blanket of organic-rich sediment over the ridge axis. Hydrothermal solutions ascend through and react with this overlying sediment, resulting in fluids that emerge from the seafloor that are enriched in Mn relative to Fe (Von Damm et al., 1985b), a condition which may favor bacterially mediated Mn(II) oxidation (Mandernack and Tebo, 1993). Indeed, a previous geochemical modeling study showed that Mn(II) oxidation rates are very fast in GB hydrothermal plumes, and scanning electron microscopy of the resulting Mn oxide particles revealed Mn oxide-encrusted bacteria, providing evidence that Mn(II) oxidation is bacterially mediated (Campbell et al., 1988).

The goal of the work presented here was to identify bacteria involved in the rapid Mn(II) oxidation and precipitation observed in GB hydrothermal sediments and plumes. Because Mn(II)-oxidizing bacteria are polyphyletic and difficult to identify based on 16S rRNA gene sequences alone, we employed culture-based methods. Many phylogenetically diverse Mn(II)-oxidizing bacteria were identified; here we report on one of the most abundantly cultured groups - spore-forming *Bacillus* species. Marine *Bacillus* spores that catalyze Mn(II) oxidation have been known for some time (Nealson and Ford, 1980; Rosson and Nealson, 1982), and many diverse *Bacillus* species produce spores that oxidize Mn(II) (Francis and Tebo, 2002). *Bacillus* sp. strain SG-1 is one of the most studied model Mn(II)-oxidizing bacteria (Bargar et al., 2005; Francis et al., 2002; Nealson and Ford, 1980; Rosson and Nealson, 1982; van Waasbergen et al., 1996; van Waasbergen et al., 1993), and is thought to oxidize Mn(II) via the multicopper oxidase MnxG (van Waasbergen et al., 1996; Webb et al., 2005a), which is deposited on the outermost layer of the spore coat (exosporium) during sporulation (Francis et al., 2002). The predicted amino acid sequence of MnxG has several short but highly conserved copper binding motifs that are characteristic of multicopper oxidases, a diverse family of enzymes that use multiple types of copper centers as cofactors in the oxidation of a wide variety of organic and inorganic compounds (Solomon et al., 1996). This study expands the previously recognized diversity of Mn(II)-oxidizing *Bacillus* spores, and is the first to identify functional genes involved in Mn(II) oxidation from a deep-sea hydrothermal vent environment.

Materials and Methods

Sample collection, strain isolation, and growth experiments. Samples were collected aboard the *R/V Atlantis* on the HOLA-1 cruise to Guaymas Basin in April and May of 2002 (Chief Scientist Jim Cowen). Hydrothermal plumes were sampled within a square area defined by 27°0'N, 111°23'W at its southeastern corner and 27°2.2'N, 111°26'W at its northwestern corner (Fig. 2.1). Plumes were detected based on turbidity anomalies as determined by a transmissometer. Plume water samples were collected in 10 L Niskin bottles on a CTD-rosette from depths ranging from 1500 m to 2000 m. Seawater to be used for culturing was transferred directly from niskin bottles to sterile 50 ml centrifuge tubes. The presence of particulate Mn oxides in hydrothermal plumes was confirmed by filtering ~10 L of water onto a 0.22 µm membrane, then adding 10 µl leukoberbelin blue (LBB) spot test reagent (Krumbein and Altmann, 1973) directly to the filter, and observing the reagent form a dark blue color. Background non-plume samples were sampled from deep waters with no turbidity anomaly. Sediment samples were collected by push cores with the *DSV Alvin*, and the temperature of the sediments was measured in situ with a titanium thermocouple on the robotic arm of *Alvin*. On board ship the surface sediments from the cores were transferred to sterile eppendorf tubes and diluted with sterile seawater. To select for spores, some samples were incubated (pasteurized) at 80°C for 20 min. Seawater and diluted sediment samples were spread on Lept, J, J-acetate, J-succinate, M, or K plates. Lept medium (Boogerd and de Vrind, 1987) contained 10 mM HEPES (pH 7.5) and 100 µM $MnCl_2$. J, M, and K media (Nealson, 1978) were modified by adding 20 mM HEPES (pH 7.8) and 100 µM $MnCl_2$

after autoclaving. J-acetate and J-succinate are J medium supplemented with 10 mM acetate or succinate. Plates were incubated at either room temperature or 4°C. Mn(II)-oxidizing strains were isolated based on the formation of brown Mn oxides on colonies, which was confirmed to be Mn oxides with LBB (Krumbein and Altmann, 1973). Cell morphology was determined by examining isolates with light microscopy; spore-forming organisms were apparent by the presence of phase-bright spores. Growth experiments were conducted in 10 ml K medium incubated with shaking at 150 rpm at room temperature, 37°C, 50°C, 55°C, or 60°C. OD at 600nm was measured every 6 to 12 hours using a Spectronic 20 spectrophotometer (Milton Roy Company).

DNA extraction, PCR, cloning, and sequencing. DNA extraction, PCR and TOPO cloning of 16S rRNA (primers 27f and 1492r) and *mnxG* (MnxGIf and MnxGIr) genes was performed as described previously (Francis and Tebo, 2002). Initially no *mnxG* PCR products were obtained from strains GB02-25, -27, and -31 but mnxG was successfully amplified by using degenerate primers MnxGUf (cagrtgratrtgctggccgat) and MnxGBr1 (raaiarrtgtrcrtgraaraa). Both strands of 16S rRNA gene PCR products were sequenced with 27f, 338f, 536r, 1074r, 1055f, and 1492r primers. *mnxG* genes were TOPO cloned into pCR2.1 and sequenced with M13f and M13r primers. Sequencing was done with either an Amersham Pharmacia Megabace 500 (SIO MBRD) or an ABI 3100 (Sequegene, Inc., and the Center for AIDS Research at UCSD) automated sequencer. All 16S rRNA gene sequences have been deposited in GenBank with the nucleotide sequence accession numbers indicated in Table 2.1. *mnxG* nucleotide sequences are are follows: DQ079011 (GB02-2A), DQ079012 (GB02-8B), DQ079013 (GB02-12),

DQ079014 (GB02-14C), DQ079015 (GB02-16), DQ079016 (GB02-21B), DQ079017 (GB02-25), DQ079018 (GB02-27), DQ079019 (GB02-30), DQ079020 (GB02-31).

Phylogenetic analysis. 16S rRNA genes were aligned with the program Sequencher and alignments were checked and edited manually. MnxG amino acid sequences were aligned with the program Clustal X (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html) using default settings with the following exceptions: for pairwise alignment parameters, gap opening was set to 1.00 and gap extension to 0.01; for multiple alignment parameters gap opening was set to 1.00 and gap extension to 0.10. PAUP (version 4.0b10) was used to generate phylogenetic trees for both 16S rRNA and MnxG sequences. Trees were constructed with neighbor joining (shown in Fig. 2.2) and maximum parsimony; both methods gave similar results.

Spore purification and Mn(II) oxidation assays. Spores were purified as described previously (Rosson and Nealson, 1982) except that the 0.15 M NaCl wash was supplemented with 0.3-1 mM ascorbate to remove Mn oxides formed during culturing. Mn(II) oxidation rate assays were performed in 500 µl reactions containing: 3 µM $CuCl_2$, 800 µM $MnCl_2$, and 20 mM HEPES pH 7.8 in milliQ-filtered water. Reactions with no spores were incubated at 4°C, room temperature, 37°C, 50°C, or 70°C for equilibration, 100 µl of purified spores were added, then incubated for 10 hours at the specified temperatures. The relative quantity of Mn oxides formed was measured by adding 50 µl of the assay to 250 µl of LBB reagent (Krumbein and Altmann, 1973) and measuring absorption at 620 nm on a Perkin Elmer Lambda Bio UV/VIS spectrophotometer. All reactions were performed in triplicate.

Results and Discussion

Guaymas Basin (GB) in the Gulf of Calfornia is a semi-enclosed basin with a sediment-covered hydrothermal system, where previous studies have suggested that bacteria play an important role in the rapid oxidation and precipitation of hydrothermally-derived dissolved Mn(II) (Campbell et al., 1988; Mandernack and Tebo, 1993). Despite the important role of bacteria in the oxidation and precipitation of Mn at GB and other deep-sea hydrothermal vents (Campbell et al., 1988; Cowen et al., 1998; Cowen and Li, 1991; Cowen et al., 1986; Cowen et al., 1990; Mandernack and Tebo, 1993), little is known about the organisms or mechanisms involved in this biomineralization. The objective of this study was to identify Mn(II)-oxidizing bacteria in GB hydrothermal sediments and plumes. Hydrothermal plumes were detected based on turbidity anomalies, and the presence of a large standing stock of particulate Mn oxide was confirmed by filtering plume water onto 0.22 μm filters. The brown particulate matter that collected on filters was determined to be Mn oxide based on the LBB test (Krumbein and Altmann, 1973) and its rapid dissolution and disappearance of the brown color upon addition of ascorbic acid. All detectable particulate Mn oxide passed through a 5 μm filter but was trapped on a 0.22 μm filter, consistent with previous observations that Mn oxides at GB are dominated by bacteria-sized particles.

Isolation and diversity of Mn(II)-oxidizing *Bacillus* spores from sediments and plumes. Mn(II)-oxidizing bacteria are known to be polyphyletic, making it difficult to identify them based on 16S rRNA sequences alone, so we focused on culture-based approaches. One of the most abundant groups of bacteria we isolated were endospore-

forming *Bacillus* species, which were cultured from high and low-temperature sediments as well as hydrothermal plumes and non-hydrothermally impacted deep waters (Table 2.1). In general the number of Mn(II)-oxidizing colonies obtained was greater from hydrothermal plumes versus non-plumes and from hydrothermal sediments versus cold sediments (Table 2.2). To specifically select for spore-forming bacteria, some sediment samples were incubated at 80°C for 20 minutes (Table 2.1). Based on microscopic and 16S rRNA sequence analaysis, all Mn(II)-oxidizing colonies examined after this treatment were spore-forming *Bacillus* species. More than 25% of the colonies recovered from hydrothermal sediments after heat treatment oxidized Mn(II), whereas only 6.7% of colonies recovered from non-hydrothermal sediments with the same heat treatment oxidized Mn(II) (Table 2.2).

Analysis of 16S rRNA genes of the Mn(II)-oxidizing spores from GB revealed that, while many of them fall into clusters defined in a previous study of Mn(II)-oxidizing *Bacillus* spores from coastal sediments (Francis and Tebo, 2002), many others represent distinct lineages (GB02-14C) and two new clusters (GB02-2A, -25, -27, and -46B; GB02-16, -18, -20, and -30) (Fig. 2.2A). Several of the isolates were found to have identical 16S rRNA genes (see Table 2.1). A number of isolates (GB02-10, 11, -12/A/B, -14B, -15, -21B/C, -26A/B/C, and -40A/B/C) from GB fell within the PL-12 cluster, a phylogenetically tight group that includes organisms and clones isolated from various environments such as the Korean traditional fermented seafood jeotgal (*B. jeotgali*), a uranium mine tailings pile, a hydrocarbon seep clone, and rice-paddy associated anoxic bulk soil ((Francis and Tebo, 2002) and references therein). Several other 16S rRNA gene sequences found in GenBank but unpublished in the literature also fall within the

PL-12 cluster, including an isolate from concretions of siderite from an arsenic-polluted aquifer in West Bengal, India (*Bacillus* sp. Cona/1), and a clone retrieved from a pink microbial mat in the Spectacles hot spring in Rehai, Tengchong, China (*Bacillus* sp. clone YJQ-55). Another group of GB isolates (GB-14A, -29, -31, -39A) forms a group just outside of the PL-12 cluster along with another clone from the hot springs in Rehai, Tengchong, China (*Bacillus* sp. clone DGG2). The isolates GB02-2A, -25, -27, and -46B form a loose group only distantly related (~97% identical) to the PL-12 cluster or any other sequences in GenBank.

GB02-14C is very different than any other previously recognized Mn(II) oxidizer, being most closely related to an uncultured soil bacterium clone 1448-1 (Valinsky et al., 2002). Four isolates (GB02-16, -18, -20, and -30) form a distinct cluster along with four species not known to oxidize Mn(II): *B. endophyticus*, an organism isolated from the inner tissues of cotton plants (Reva et al., 2002); *Bacillus* sp. 19490, an isolate from a biodeteriorated mural painting (Heyrman and Swings, 2001); and *Bacillus* sp. strain SCD-2001, a fluorescent soil isolate (Magyarosy et al., 2002). Three of these Mn(II)-oxidizing isolates (GB02-16, -18, and -20) have identical 16S rRNA gene sequences and were isolated on the freshwater medium LEPT, but will also grow on the seawater-based K medium. Five GB isolates (GB02-5, -8A/B/C/D) have identical 16S rRNA gene sequences that fall within the SG-1 cluster.

mnxG, a multicopper oxidase known to be involved in Mn(II) oxidation in *Bacillus* sp. strain SG-1 (van Waasbergen et al., 1996; Webb et al., 2005a) and other Mn(II)-oxidizing *Bacillus* strains (Francis and Tebo, 2002), was amplified from all GB Mn(II)-oxidizing isolates, indicating that these deep-sea hydrothermal vent isolates likely

share a copper-dependent mechanism of Mn(II) oxidation with previously characterized Mn(II)-oxidizing *Bacillus* spores. *mnxG* could not be amplified from non-Mn(II) oxidizing *Bacillus* spores (Francis and Tebo, 2002) and *mnxG* is not present in genomes of any non-Mn(II)-oxidizing *Bacillus* species sequenced to date, suggesting that *mnxG* is a good genetic marker for the ability of *Bacillus* spp. to oxidize Mn(II). We were unable to amplify *mnxG* with primers mnxGIf and mnxGIr from several of our Mn(II)-oxidizing isolates, so we designed new degenerate primers targeting conserved copper-binding regions. The degenerate primers yielded good amplification of *mnxG* from diverse Mn-oxidizing spore formers, suggesting that these primers may be suitable for amplifying *mnxG* directly from environmental DNA in order to gain a culture-independent view of the diversity of this gene in the environment.

Phylogenetic trees constructed based on predicted MnxG amino acid sequences (Fig. 2.2B) are very similar to those based on 16S rRNA genes (Fig. 2.2A), consistent with there having been no extensive horizontal transfer of *mnxG* genes in these organisms (Francis and Tebo, 2002). However, there are a few exceptions among the GB isolates. Based on 16S rRNA, GB02-2A falls near the PL-12 cluster (Fig. 2.2A), whereas the MnxG phylogeny places it with the GB02-16 cluster (Fig. 2.2B). Also, the MB-9 MnxG sequence does not group with the "halo" cluster as it does by 16S rRNA. The "halo"cluster is a group of organisms so named because of the ability of its members to grow under high salt conditions (Francis and Tebo, 2002). In view of MB-9's lower tolerance for salt (Francis and Tebo, 2002) and its only loose phylogenetic affiliation, it seems that this isolate should not be considered a member of the "halo" cluster. More phylogenetic analysis with additional genes is required to resolve the exact phylogenetic

relationships among the deepy branching Mn oxidizers (MB-9) and those for which 16S rRNA and MnxG sequences give different results (GB02-2A).

Growth and Mn(II) oxidation properties of *Bacillus* isolates. Due to the ability of *Bacillus* spores to persist under harsh conditions over long periods of time, it is unclear whether the GB isolates are metabolically active as vegetative cells in the sediments and plumes where we isolated them, or if they are deposited to these areas with sinking organic matter from surface waters or river runoff. To address this question, we determined the growth characteristics of a selection of isolates over a range of temperatures. Several strains (GB02-8B, -27) clearly had faster initial growth rates at 50°C than at 37°C, although higher final growth yields were usually observed at 37°C, indicating mesophily or perhaps slight thermophily (data not shown). Other isolates grew best at room temperature or 37°C (e.g. GB02-12). These results indicate that while the GB Mn(II)-oxidizing *Bacillus* isolates may be able to grow at some of the moderately hot sediments sampled, they were most likely present only as spores in sediment samples above 60°C (see Table 2.1). Spore-forming *Bacillus* species capable of Mn(II) oxidation seem to be able to grow over a wide range of temperature and salt concentrations (Francis and Tebo, 2002), making it difficult to infer whether these organisms are specifically adapted to marine and/or hydrothermal environments.

Regardless of whether the *Bacillus* isolates are metabolically active in GB hydrothermal sediments and plumes, Mn(II)-oxidizing activity is associated with the spore and is therefore likely to be active over a wide range of conditions. To verify that Mn(II)-oxidizing activity could occur at high temperatures, we assayed Mn(II) oxidation by purified spores of several isolates at 4, 25, 37, 50, and 70°C. The activity versus

temperature profile varied for different spores, but there was always significant activity at 50°C and usually at 70°C (Fig. 2.3). Taken together with their growth characteristics (mesophilic not thermophilic), this data suggests that the *Bacillus* species isolated here are present as active Mn(II)-oxidizing spores at the sediment-water interface of hydrothermal sediments. Therefore, Mn(II)-oxidizing *Bacillus* spores in surface sediments might play an important role in oxidizing Mn(II), perhaps contributing to the short residence time of dissolved Mn in the Guaymas Basin.

Mn-Oxidizing Spores from other Deep-Sea Hydrothermal Vents. Because GB represents a unique deep-sea hydrothermal vent environment, we sequenced the 16S rRNA genes from several Mn(II)-oxidizing bacteria from our culture collection isolated on previous expeditions to hydrothermal vents from the Juan de Fuca ridge (Tebo, unpublished results). Two of these isolates were spore-forming *Bacilli*; one fell within the PL-12 cluster, and one within the SG-1 cluster. The third isolate was also a gram positive bacterium, most closely related to *Microbacterium oxydans* (Schumann et al., 1999). Results from our laboratory indicate that Mn(II)-oxidizing spores of *Bacillus* species can also be isolated from Vailulu'u (A. Templeton and Tebo, unpublished results), an underwater volcano in the South Pacific (Staudigel et al., 2004). Thus Mn(II)-oxidizing *Bacillus* spores seem to be widespread at deep-sea hydrothermal vent environments.

Our results show that diverse Mn(II)-oxidizing *Bacillus* spores with *mnxG* multicopper oxidase genes can be isolated from GB hydrothermal sediments and plumes, extending the previously recognized diversity of Mn(II)-oxidizing *Bacillus* spores and *mnxG* genes. Although there have been several brief descriptions of the isolation of

Mn(II)-oxidizing bacteria from deep-sea hydrothermal vents (Durand et al., 1990; Ehrlich, 1983; Ehrlich, 1985), this study provides the first 16S rRNA gene-based identification of such bacteria. The *mnxG* multicopper oxidase genes reported here are the first functional genes involved in Mn(II) oxidation to be recovered from deep-sea hydrothermal vents, where bacteria are thought to play a crucial role in mediating rapid Mn(II) oxidation and precipitation of Mn oxides on massive, globally distributed scales. Mn(II)-oxidizing *Bacillus* spores show extremely rapid and stable Mn(II)-oxidizing activity and their presence in GB hydrothermal sediments and plumes could significantly contribute to the rapid Mn(II) oxidation that takes place at GB.

Bacillus species are generally recognized as being ubiquitous in many environments and have been isolated from marine sediments (Bonde, 1981; Siefert et al., 2000) and several deep-sea hydrothermal vents including Guaymas Basin (Caccamo et al., 2001; Marteinsson et al., 1996), but few studies that we are aware of have addressed the numerical or biogeochemical significance of *Bacillus* spores in the marine environment – especially in the water column. One possible explanation for the presence of *Bacillus* spp. in the water column at GB is that they are attached to suspended particulate matter (SPM) (Campbell and Gieskes, 1984) that could include fine sediment particles entrained by ascending hydrothermal fluids. Previous work in our laboratory demonstrated that a significant fraction of Mn(II)-oxidizing activity in coastal San Diego sediments is heat resistant, consistent with catalysis by a spore coat enzyme (Lee, 1994). More cultivation-independent studies using molecular and biogeochemical approaches are needed to determine whether *Bacillus* spores such as those identified here

significantly impact Mn biogeochemistry in Guaymas Basin and other deep-sea hydrothermal vent environments.

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Table 2.1. Mn(II)-oxidizing *Bacillus* strains used in this study

Accession	Isolate	Isolated from[b]	Medium	Temp°C
DQ079008	GB02-2A	Mat-covered sediment	K	4-31
DQ078996	GB02-5	Mat-covered sediment (HT)[c]	K	4-31
DQ078996	GB02-8A/B/C/D (4)[a]	Mat-covered sediment	K-vpw[d]	75-82
DQ078999	GB02-10/11 (2)[a]	Non-plume water; depth = 486m	M	3
DQ079005	GB02-12/A/B (3)[a]	Black hydrothermal sediment	K-vpw[d]	24-66
DQ079001	GB02-14A	Sediment (HT)[c]	M	3
DQ078995	GB02-14B	Sediment (HT)[c]	M	3
DQ079004	GB02-14C	Sediment (HT)[c]	M	3
DQ078999	GB02-15	Plume; depth = 1812 m	M	3
DQ079006	GB02-16/18/20 (3)[a]	Sediment (HT)[c]	LEPT	3
DQ078998	GB02-21B/C (2)[a]	Plume; depth = 1812 m	M	3
DQ079010	GB02-25	Mat-covered sediment	K	4-31
DQ078998	GB02-26A/B/C (3)[a]	Mat-covered sediment	M-vpw[d]	75-82
DQ078997	GB02-27	Sediment (HT)[c]	K	3
DQ079002	GB02-29A/B/C/D (4)[a]	Sediment (HT)[c]	K/J-ace	3
DQ079007	GB02-30	Sediment (HT)[c]	K	3
DQ079000	GB02-31	Sediment (HT)[c]	K	3
DQ079003	GB02-39A	Sediment (HT)[c]	M	3
DQ078998	GB02-40A/B/C (3)[a]	Mat-covered sediment (HT)[c]	M	75-82
DQ079009	GB02-46B	Sediment (HT)[c]	K	3

[a]Numbers in parentheses indicate the number of isolates that were sequenced and found to have identical 16S rRNA gene sequences.
[b]All sediment samples were collected at a depth of ~ 2000 m.
[c]HT indicates that samples were treated at 80°C for 20 minutes prior to plating.
[d]vpw indicates that the media was made with GB vent plume water.
[e]Temp refers to the temperature of the sample upon collection.

Table 2.2. Plate Counts of Mn(II)-oxidizing Bacteria

	# Mn oxide encrusted colonies	# total colonies	% colonies oxidize Mn
Plumes	146	2250	6.5
Non-Plumes	3	359	1.9
Hydrothermal Sediments	144	1248	11.5
Non-Hydrothermal Sediments	91	2188	4.1
Pasteurized[a] Hydrothermal Sediments	132	523	25.2
Pasteurized[a] Non-Hydrothermal Sediments	41	608	6.7

[a]80°C for 20 minutes.

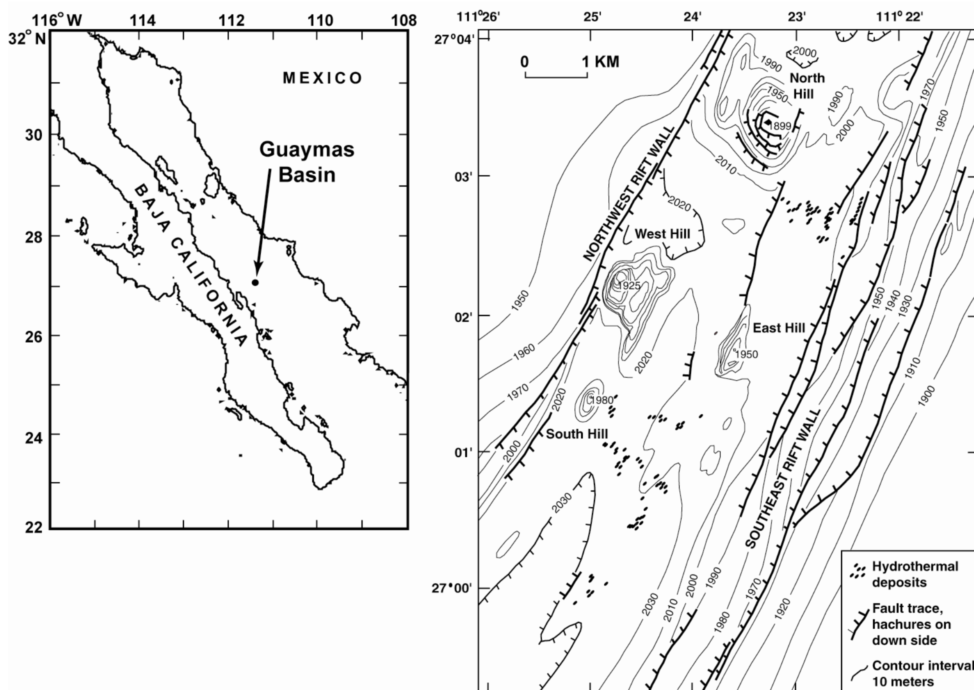

Figure 2.1. (a) Location of the study site, Guaymas Basin in the Gulf of California. (b) Detailed sketch map of the seafloor features at the study site, adapted from reference 23.

Figure 2.2. Unrooted neighbor-joining phylogenetic trees based on 16S rRNA sequences (A) and MnxG amino acid sequences (B). Mn(II)-oxidizing isolates from Guaymas Basin are indicated by boldface type and are designated "GB02-". An asterisk indicates that multiple isolates were found to have identical 16S rRNA gene sequences (Table 2.1). Mn(II)-oxidizing isolates from coastal San Diego sediments are indicated by gray type. Additional Bacillus species, isolates, and clones are not thought to be capable of Mn(II) oxidation are are included for reference. Bootstrap values based on 1,000 replicates (>65) are indicated at the branch points.

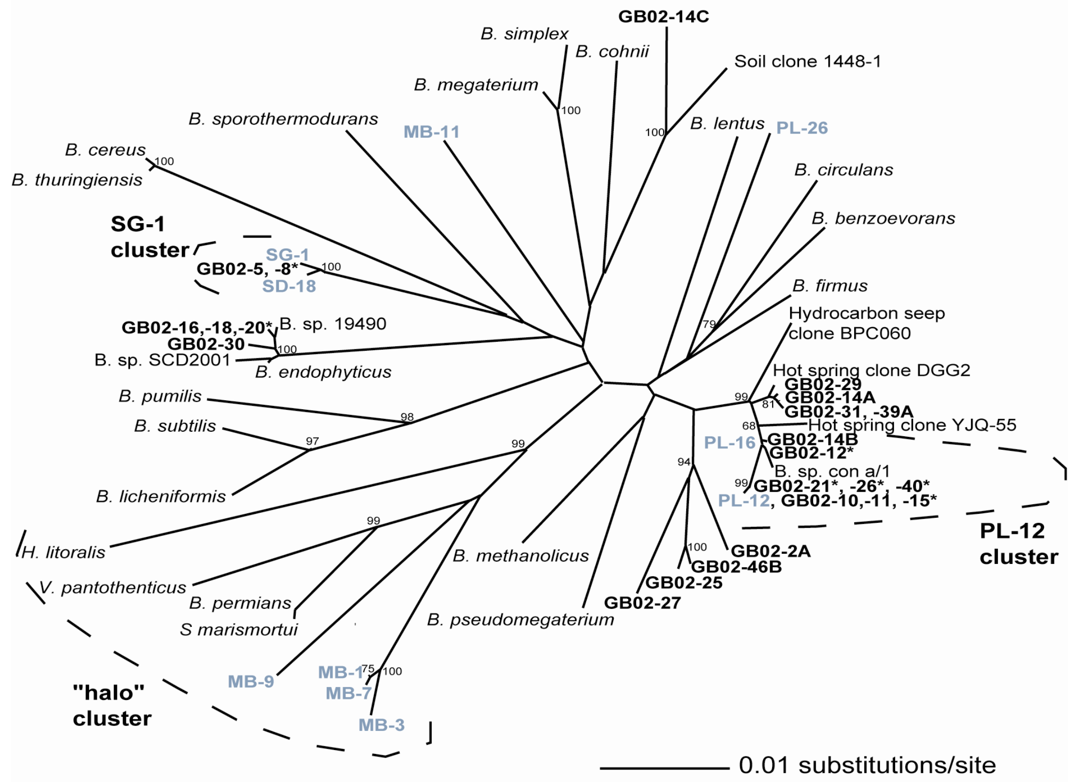

Figure 2A
GB02-14C
B. simplex
B. cohnii
Soil clone 1448-1
B. megaterium
100
B. sporothermodurans
MB-11
100
B. lentus
PL-26
B. cereus
100
B. thuringiensis
B. circulans
B. benzoevorans
SG-1
cluster
SG-1
GB02-5, -8*
100
SD-18
B. firmus
Hydrocarbon seep
clone BPC060
79
GB02-16,-18,-20*
B. sp. 19490
GB02-30
100
B. sp. SCD2001
B. endophyticus
Hot spring clone DGG2
GB02-29
GB02-14A
GB02-31, -39A
Hot spring clone YJQ-55
B. pumilis
98
B. subtilis
97
PL-16
GB02-14B
GB02-12*
B. sp. con a/1
GB02-21*, -26*, -40*
PL-12, GB02-10,-11, -15*
B. licheniformis
99
PL-12
cluster
H. litoralis
99
100
GB02-2A
GB02-46B
GB02-25
GB02-27
B. methanolicus
V. pantothenticus
B. permians
S marismortui
B. pseudomegaterium
"halo"
cluster
MB-9
MB-1
75
100
MB-7
MB-3
0.01 substitutions/site

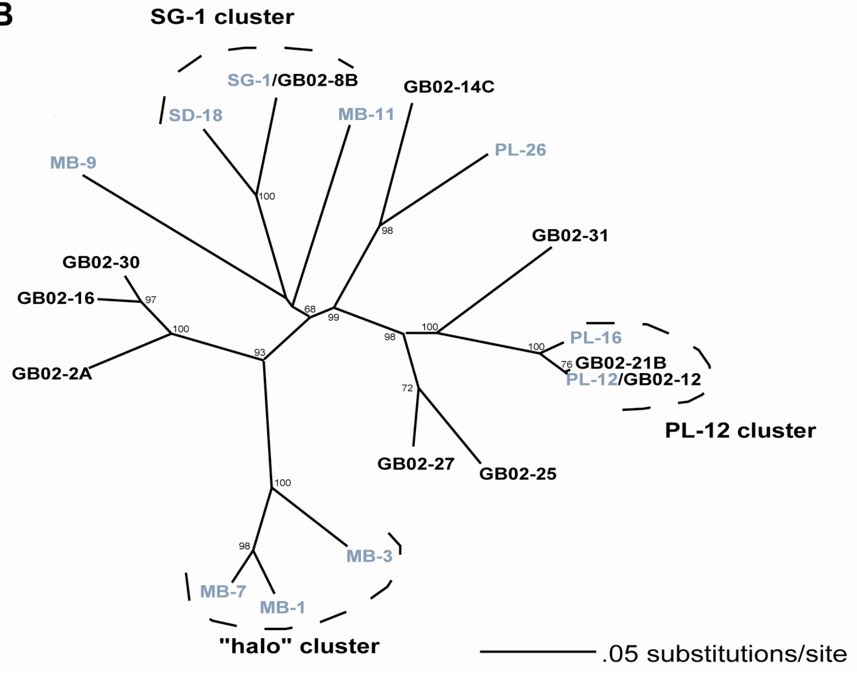

Figure 2B
SG-1 cluster
SG-1/GB02-8B
GB02-14C
SD-18
MB-11
PL-26
MB-9
100
98
GB02-31
GB02-30
GB02-16
97
100
68
99
100
98
GB02-2A
93
PL-16
100
76
GB02-21B
PL-12/GB02-12
72
PL-12 cluster
GB02-27
GB02-25
100
98
MB-3
MB-7
MB-1
"halo" cluster
.05 substitutions/site

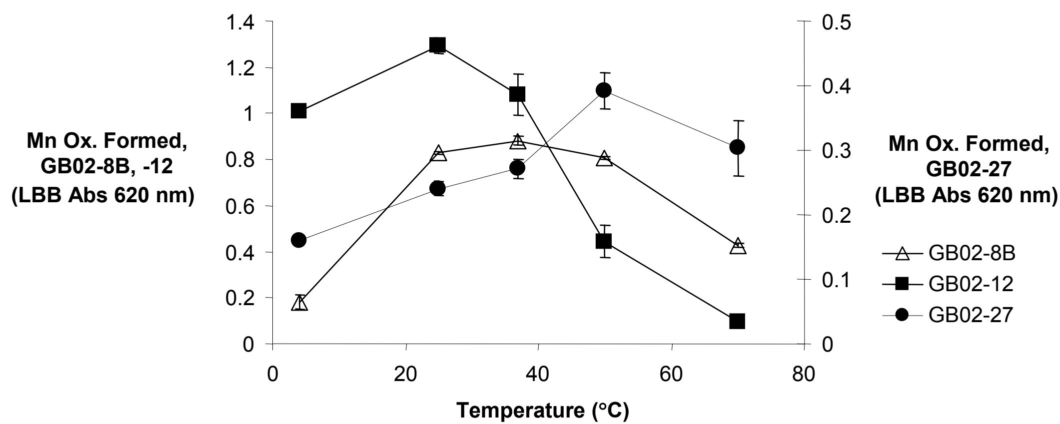

Figure 2.3. Relative amounts of Mn oxidized at different temperatures by Bacillus spores isolated from Guaymas Basin. Mn Ox., Mn oxide; Abs 620 nm, absorbance at 620 nm.

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III

Enzymatic microbial Mn(II) oxidation in the Guaymas Basin hydrothermal plume

Abstract

At Guaymas Basin (GB) in the Gulf of California, hydrothermal venting injects fluids laden with dissolved Mn(II) (dMn) into the deep waters of the basin. In hydrothermal plumes, the dMn is oxidized and precipitated as particulate Mn oxides, generating turbid “hydrothermal clouds”. Previous studies have suggested that this dMn removal at GB is extremely fast and therefore must be the result of microbially-mediated Mn(II) oxidation and precipitation. Here we show that dMn removal rates at GB are the fastest ever observed in a deep-sea hydrothermal plume (up to 2.52 nM/hr), and confirm that microbial oxidation of Mn(II) plays a crucial role. dMn removal is dramatically inhibited under anoxic conditions and by the presence of the poison sodium azide. dMn removal rates in background deep-sea water – either above the GB plume or at GB plume depths (~1750 – 2000 m) in the non-hydrotheramlly impacted Carmen Basin – are > 20 times slower than in GB. We suggest that this rapid plume-specific dMn oxidation reflects a unique microbial community within the plume. dMn removal rates display a distinct temperature optimum at ~40°C and a saturation or Michaelis-Menten like response to O_2 concentration, consistent with an enzymatic mechanism. dMn removal was resistant to heat treatment used to select for spore-forming organisms, but very sensitive to small additions of $CuCl_2$. Overall, these results reveal a vigorous Mn biogeochemical cycle in the deep-sea hydrothermal plume of GB, where a distinct microbial community enzymatically catalyzes rapid Mn(II) oxidation and precipitation.

Introduction

Manganese (Mn) plays an important role in the biogeochemistry of the oceans, both in the soluble state as a micronutrient for organisms and in the oxidized, solid state as a scavenger of other trace elements. The oxidized solid-phase Mn oxides are strong scavengers and reactive oxidants that have earned the nickname "the scavengers of the sea" (Goldberg, 1954). Deep-sea hydrothermal vents are a significant source of Mn for the world's oceans (Edmond et al., 1982). Reducing hydrothermal fluids, often enriched in Mn greater than a million times over ambient seawater, are injected into the deep-sea at vents along mid-ocean ridges, forming hydrothermal plumes that can rise hundreds of meters above the seafloor and be transported thousands of kilometers from their source. In hydrothermal plumes, hydrothermal fluids mix with oxygenated deep-seawater, and dMn is scavenged onto particles and/or oxidized and precipitated to form Mn oxide minerals. These particles sink towards the seafloor, scavenging trace elements and contributing to metalliferous sediments (Edmond et al., 1982). Because of its high concentration in hydrothermal plumes relative to background seawater, Mn is a valuable and commonly used tracer of hydrothermal activity (Baker et al., 1995; Lilley et al., 1995).

The conversion of dMn to the particulate phase may occur by several processes, including adsorption onto particles or cells, uptake into cells, or oxidative precipitation as Mn oxides. We use the term "dMn removal" to include all of these processes, however Mn(II) oxidation is thought to be the dominant process at oxic/anoxic interfaces where

Mn cycling is prevalent (Sunda and Huntsman, 1987; Tebo and Emerson, 1985; Tebo et al., 1991). The chemical oxidation of Mn(II) by O_2 is thermodynamically favorable but kinetically slow, and in many environments microorganisms catalyze Mn(II) oxidation at rates that are orders of magnitude faster than those due to abiotic processes (Tebo et al., 2004). At deep-sea hydrothermal vents, microorganisms have been implicated in dMn removal by several lines of evidence: (1) inhibition of dMn removal by poisons such as sodium azide (Cowen et al., 1998; Cowen et al., 1986; Cowen et al., 1990; Mandernack and Tebo, 1993), (2) observation of Mn-encrusted microbial cells by transmission electron microscopy (TEM) (Campbell et al., 1988; Cowen et al., 1998; Cowen et al., 1986), and (3) isolation of Mn(II)-oxidizing bacteria from vent sites (Dick et al., 2006; Ehrlich, 1983; Ehrlich, 1985). Despite the importance of microorganisms in mediating dMn removal at deep-sea hydrothermal vents, little is known about the molecular or biochemical pathways involved. In Mn(II)-oxidizing bacteria that have been studied in culture, Mn(II) oxidation is enzymatic (Francis et al., 2001; Francis and Tebo, 2002; Rosson and Nealson, 1982), but evidence that this is the operative mechanism in deep-sea hydrothermal plumes is lacking.

Mandernack and Tebo (1993) showed that dMn removal rates and the relative contribution of microbial activity versus chemical processes (e.g. adsorption or coprecipitation with iron oxyhydroxides) vary dramatically among different hydrothermal vent sites. At the Galapagos spreading center microbial activity was very important, but at the Endeavor Ridge (Juan de Fuca spreading center) Mn scavenging occurred primarily via an abiotic mechanism (Mandernack and Tebo, 1993). Guaymas Basin (GB), located in the Gulf of California (Fig. 3.1), is an oft-cited example of a deep-sea hydrothermal

system where microbially-mediated dMn removal dominates. This reputation is based on a geochemical modeling study that calculated (based on the flux and standing stock of dMn) extremely short residence times of dMn, and found that Mn oxide particles at GB are dominated by Mn-encrusted microorganisms (Campbell et al., 1988). dMn removal experiments have been reported for only one GB sample, but they showed a high turnover rate and short residence time for dMn, and removal was dominated by oxidation rather than adsorption (Mandernack and Tebo, 1993). However, no studies have directly measured dMn removal rates at GB or analyzed the importance of microorganisms in catalyzing them. In this study, we confirm that GB Mn removal rates are extremely rapid and catalyzed by microorganisms, and investigate the nature of the microbial population and biochemical mechanism(s) that drive this vigorous Mn cycle.

Geological Setting and Site Description. Guaymas Basin (GB) is one of a series of deep basins that occur along the Central and Southern Gulf of California (Fig. 3.1). The formation of the Gulf and its deep basins has been driven by sea floor spreading. This rifting gives rise to hydrothermal activity, but based on ^{3}He and silica data, only GB is thought to be hydrothermally active (Campbell and Gieskes, 1984; Lupton, 1979). GB contains two spreading centers that occur in troughs; hydrothermal activity is best documented in the Southern Trough (Lonsdale and Becker, 1985) and subsequent submersible dives, exploration and experimental work over the past two decades have been focused there.

GB is an unusual setting for a deep-sea hydrothermal vent system due its location in a basin and its proximity to the coast. Because hydrothermal fluids are injected into the deep waters of a semi-enclosed basin, inputs accumulate to higher concentrations than

at mid-ocean ridges, where venting occurs at topographic highs into the open ocean. GB sits under productive surface waters (van Andel, 1964), and high sedimentation rates have blanketed the spreading axis with a ~400 m layer of sediment (Lonsdale and Becker, 1985). Reactions between this sediment layer and ascending hydrothermal fluids influence the chemistry of hydrothermal fluids that emerge from the seafloor. In particular, the pH is raised, resulting in precipitation and removal of sulfides of metals such as Fe. dMn is more stable at higher pH and remains in solution to a greater extent than Fe, thus GB hydrothermal fluids have an unusually high Mn:Fe ratio ranging from 1.25 to 10, with dMn concentrations of 128-236 μmoles/kg (Von Damm et al., 1985b). Hydrothermal inputs are the major source of Mn for the basin (Campbell et al., 1988). In the plume, it is rapidly converted to a particulate phase consisting of 2-5 μm particles that resemble Mn-encrusted microbes and are responsible for a turbid "hydrothermal cloud" that sits in the basin (Campbell et al., 1988) and is easily detectable with a transmissometer.

Materials and Methods

Sample collection. Samples from Guaymas Basin (GB) were collected aboard the *R/V New Horizon* on three cruises: GoCAL1 (July 2004), GoCAL2 (January/February 2005), and GoCAL3 (July/August 2005). GB was sampled on all three cruises; 11 "tow-yo" casts (see below) were done in the Southern Trough (station 1) and 10 tow-yos in the Northern Trough (station 2). Two vertical casts were done at Carmen Basin (CB) (station 3) on GoCAL3. Station locations are shown in Fig. 3.1. Hydrothermal plumes were

detected by turbidity anomalies as measured by an air-calibrated transmissometer (WetLabs) on a CTD rosette (Sea-Bird). Plumes were mapped by tow-yo (Baker et al., 1995), whereby the CTD is lowered and raised from ~ 1700 – 2000 m depth while being dragged by the ship at a speed of ~ 1 knot. All samples were collected in 10 L niskin bottles.

Determination of dissolved and total Mn concentrations. Samples were transferred from niskin bottles to acid-washed 50-ml polypropylene tubes. Samples for dMn were filtered through 0.2 μm acid-washed nucleopore polycarbonate filters within 1 hour of collection. Filtrate (dMn) and unfiltered total Mn samples (tMn) were stabilized by acidification with Optima grade nitric acid to a pH of < 2 and stored at 4°C until analysis. All shipboard manipulations were performed in a laminar flow hood with clean techniques. Mn concentrations were determined on a Thermoquest Finnigan Element 2 double focusing, single collector, magnetic sector inductively coupled plasma mass spectrometer (ICP-MS) at the SIO unified laboratory analytical facility. ICP-MS was done at low resolution following instrument and induction parameters described previously (Field et al., 1999). Samples were diluted 1:50 in 2% nitric acid in quartz-distilled (QD) water prior to analysis. A calibration curve was prepared as described previously (Rodushkin and Ruth, 1998) using matrix-matched external standards made with 2% natural seawater stripped of metals by precipitation with Optima ammonium hydroxide. Indium (1 ppb) was used as an internal standard in all standards and samples. Standard additions (Willard et al., 1965) were used to rule out a matrix effect. To confirm analytical accuracy, reference waters CASS-4 and NASS-5 (Verplank et al., 2001) were included in the analysis as samples. Our experimentally determined average

Mn concentration for CASS was 56 +/- 10 nM (reported to be 51 nM) and for NASS-5 it was16 +/- 8 nM (reported to be 17 nM). Six samples from station 1 were collected, processed and analyzed in duplicate. The average standard deviation was 8 nM, which includes variation due to both sampling and analytical error.

dMn removal experiments. The general experimental design for determining dMn removal rates was as described previously (Mandernack and Tebo, 1993; Sunda and Huntsman, 1987; Tebo et al., 1991). Experiments were performed on-deck using $^{54}Mn(II)$ as a radioactive tracer to measure the conversion of dMn to the particulate phase, as defined by retention on a 0.2 μm pore-size filter. Water samples were transferred from niskin bottles to the radiation van in polycarbonate bottles (0.25 – 4 L), and care was taken to keep them cold at $< 4°C$ at all times. Incubations were typically started within 4 hours of sample collection and always within 12 hours. For time course experiments, 100-ml of sample were transferred to acid-clean 250-ml polycarbonate bottles, and amended with 0.14 - 0.26 μCi carrier free $^{54}MnCl_2$. Time courses were done in replicate bottles, with duplicate 5-10 ml samples from each bottle filtered and processed at each time point as described below. Rates were determined based on the linear part of the curve, usually the first 4-6 hours. For end-point experiments (depth profiles, anoxic, and desorption experiments), 40-ml of sample were transferred to acid-clean 60-ml polycarbonate bottles, and amended with 0.010 to 0.026 μCi $^{54}MnCl_2$. End point experiments were done in triplicate bottles, and incubation times ranged from 4-10 hours. All incubations were done at 4°C (except temperature optimum experiments described below). The quantity of ^{54}Mn converted to the particulate phase was

determined by vacuum filtration through 0.2 μm multicellulose ester membrane filters. After filtration of sample, filters were rinsed with 3-5 ml 0.2 μm-filtered seawater. ^{54}Mn was counted from three fractions – total (sampled before filtration), soluble (filtrate), and particulate (filter) – so that mass balance could be calculated. To dissolve and evenly disperse particulate Mn prior to counting, 1 ml 0.1% hydroxylamine hydrochloride (H-HCl) was added to 2 ml total samples, and 3 ml H-HCl was added to filters. Samples (3 ml) were counted on a LKB Wallac 1282 Compugamma CS Universal (Perkin Elmer) gamma counter. The portion of ^{54}Mn retained on the filter over time as a fraction of the total gives the fraction of ^{54}Mn removed, which multiplied by dMn concentration equals the dMn removal rate.

The important processes and mechanisms of dMn removal were assessed by manipulating ^{54}Mn incubation experiments under a variety of conditions. Biological processes were assessed by including the poison sodium azide at a concentration of 0.1% (w/v). To determine the fraction of ^{54}Mn present as (or scavenged by) extracellular Mn oxide, ascorbic acid (pH adjusted to 8 with NaOH), an efficient reductant of Mn oxide, was added to replicate bottles to a final concentration of 40 μM at the end of incubations (30 minutes prior to filtration). Anoxic experiments were performed in acid-clean 60 ml glass serum bottles with butyl stoppers sealed with aluminum caps. Prior to addition of ^{54}Mn, serum bottles were bubbled with N_2 and CO_2 using a gas proportioner to maintain pH at 7.6 – 7.8 as described previously (B.G. Clement et al., in preparation). Desorption experiments were conducted by the addition of cold (nonradioactive) $MnCl_2$ to exchange with bound ^{54}Mn (Burdige and Nealson, 1986; Mandernack and Tebo, 1993; Myers and Nealson, 1988; Sunda and Huntsman, 1987). After initial filtration of sample, the

vacuum was broken and the filter was overlaid with 3 ml $MnCl_2$ in filtered seawater, which was then filtered through after 15 minutes ("Mn rinse"). We tried several different concentrations of $MnCl_2$ including 100 μM, 1 mM, and 10 mM. In one experiment, desorption was also investigated by adding $MnCl_2$ directly to the incubation bottles (to a final concentration of 10 mM) 15 minutes prior to filtration ("Mn addition"). For copper addition experiments, $CuCl_2$ was added at the beginning of incubations, prior to addition of ^{54}Mn. Temperature optimum experiments were done by pre-equilibrating samples to the appropriate temperature (4, 20, 40, 55, 70°C) in water baths prior to addition of $^{54}MnCl_2$. After addition of tracer, the bottles were quickly returned to water baths. For heat treatment experiments, samples were incubated in a water bath at 80°C for 20 min, transferred to a 4°C water bath for equilibration, then spiked with $^{54}MnCl_2$ and incubated at 4°C for the remainder of the experiment.

Results and Discussion

General water column features and location of hydrothermal plumes. During a series of three cruises aboard the *R/V New Horizon*, we investigated the rates and mechanisms of dMn removal from both spreading troughs of GB and its southern neighbor, Carmen Basin (CB), which is not thought to be impacted by hydrothermal activity (Fig. 3.1). Hydrothermal plumes were sampled by tow-yo of the CTD (Baker et al., 1985), and had profiles of dissolved and particulate Mn, oxygen, and turbidity similar to those observed previously (Fig. 3.2) (Campbell et al., 1988). In general, more dMn and turbidity structure was observed in the Southern Trough, above areas of previously

described hydrothermal venting. The strongest plume signal was encountered at station 1 at a depth of 1996 m (dMn = 258 nM, tMn = 356 nM). Northern Trough profiles were less dynamic and typically featured increasing Mn and turbidity with depth.

Dissolved Mn removal rates and processes. Time course measurements of dMn removal rates in the GB hydrothermal plume demonstrated a generally linear dMn removal rate for the first 4-6 hours (Fig. 3.3) that ranged from 0.43 to 2.52 nM/hr (Table 3.1). The fastest rates were measured in areas directly above known sites of hydrothermal venting in the Southern Trough (1-2 #3, 1-3 #1). However, comparable rates were also recorded in the Northern Trough, away from known vent fields (2-27a #1, 2-10 #11). dMn removal rates were measured shipboard at atmospheric pressure and so may represent an underestimate of the *in situ* rate, which has been shown to be enhanced at *in situ* pressure conditions (Cowen, 1989; Mandernack and Tebo, 1993). Nevertheless, the dMn removal rates we report here are the fastest to be measured in a deep-sea hydrothermal plume by at least a factor of 20. Previously reported dMn removal rates from other vent environments include (in nM/hr): 0.0004 – 0.1004 at the Galapagos spreading center (Mandernack and Tebo, 1993), 0.0002 – 0.1033 at the Endeavor Ridge (Mandernack and Tebo, 1993), and 0.0002 – 0.0023 at the Cleft Segment (Cowen et al., 1990) (both of the Juan de Fuca spreading center). Residence time for dissolved Mn was calculated from:

$$\tau = A_{Mn}/[d\mathrm{dMn}/dt]$$

where A_{Mn} is the dMn concentration and ddMn/dt is the measured dMn removal rate. Residence time varied from 10-86 hours (Table 3.1), fitting within an upper limit for residence time (1-2 weeks) predicted by a geochemical model (Campbell et al., 1988),

but shorter than the 26 days previously calculated from one GB sample (Mandernack and Tebo, 1993). The dMn residence times we calculate for GB are much shorter than those of the open ocean (Landing and Bruland, 1987) or some hydrothermal vent environments (Mandernack and Tebo, 1993), which are on the time scale of years. But comparable residence times have been recorded in other areas of intense Mn cycling, such as the Galapagos spreading center (as low as 28 days) (Mandernack and Tebo, 1993), and the oxic/anoxic interfaces of Saanich inlet (2-5 days) (Emerson et al., 1982) and the Black Sea (as low as 0.6 days) (Tebo, 1991).

Extremely rapid dMn removal rates are indicative of catalysis by microorganisms. To directly assess the contribution of microbial activity, we assayed the dMn removal rate in the presence of sodium azide, thought to be a suitable poison for microbially-mediated Mn removal because it interferes little with Mn solution chemistry (Emerson et al., 1982; Rosson et al., 1984). The presence of azide dramatically inhibited the dMn removal observed in untreated samples (Fig. 3.3), suggesting a biological mechanism. There was an initial removal observed in the presence of azide, which likely reflects equilibration of the ^{54}Mn(II) tracer with sorption sites on particles.

To determine if the biologically-mediated dMn removal was due to precipitation on cellular surfaces or uptake into the cell, we treated replicate bottles with ascorbic acid, a strong reducing agent that readily dissolves Mn oxides (Stone, 1983; Sunda and Huntsman, 1987) but does not lyse cells (Anderson and Morel, 1982). Addition of ascorbic acid after termination of the experiment completely reduced all particulate Mn, demonstrating that dMn removal was due to extracellular oxidation or adsorption onto preexisting Mn oxides rather than intracellular uptake (data not shown). To further assess

the importance of oxidation of Mn(II) to MnO_2 versus the adsorption of Mn(II) onto preexisting Mn oxides, dMn removal experiments were performed under anoxic conditions or with rinses/additions of "cold" (nonradioactive) $MnCl_2$ to desorb Mn(II). Most dMn removal (59-73%) was inhibited under anoxic conditions (Table 3.2), indicating that O_2 is required for the majority of dMn removal. 0-33% of ^{54}Mn(II) was displaced by cold $MnCl_2$ rinses or additions, consistent with sorption being a potentially significant but not the major factor in dMn removal (Table 3.2). Taken together, our dMn removal experiments indicate that biologically-mediated, extracellular oxidation and precipitation of Mn oxides is the primary process responsible for dMn removal in the GB hydrothermal plume.

Depth profiles of dMn removal rates in the Guaymas and Carmen Basins. Productive surface waters in the Gulf of California lead to sedimentation rates that are about two orders of magnitude higher than in the open ocean (van Andel, 1964). This elevated flux of organic material has been suggested to sustain the high microbial Mn(II)-oxidizing activity in GB waters (Campbell et al., 1988; Mandernack and Tebo, 1993). To determine whether fast dMn removal rates are a common feature throughout the GB water column, we measured dMn removal across a range of depths. Fast dMn removal rates were observed only at the depths of the hydrothermal plume as identified by a minimum in light transmission (Fig. 3.2A). Similar rates were observed in the Northern and Southern Troughs (Table 3.1; Fig. 3.2A, B). Hydrothermal deposits have been recovered from the Northern Trough (Lonsdale et al., 1980) however previous work detected no plumes there (Lonsdale and Becker, 1985). We recorded Mn concentrations and dMn removal rates in the Northern Trough that were comparable to those of the

Southern Trough. It is not known whether these currently elevated Mn concentrations of the Northern Trough are indicative of hydrothermal inputs at that site or spillover from the Southern Trough.

Due to the semi-enclosed nature of GB, hydrothermal inputs are retained and there is no background deep seawater that has not been impacted by hydrothermal activity. Carmen Basin (CB), the next deep basin south of Guaymas, is not thought to have hydrothermal activity (Campbell and Gieskes, 1984; Lupton, 1979) and thus should provide a non-plume control for the depths of the GB plume. Water column data for CB are shown in Fig. 3.2C. A slight light transmission anomaly is present at just below 1500 m, which likely represents spillover of GB plume water at the sill depth (1560 m). Between ~1750 – 2000 m (the depth interval of the GB plume), light transmission is higher and the Mn concentrations are much lower than that of GB. These data are consistent with the other deep basins of the Gulf of California, which are not influenced by hydrothermal activity (Campbell et al., 1988). At these depths, dMn removal rates are also consistent with background seawater observed throughout the water column, indicating that the rapid rates observed at 1750 – 2000 m depth in GB are specific to the hydrothermal plume rather than to those depths. Below 2000 m, Mn concentrations begin to increase, and there are turbidity, dMn and dMn removal maxima at ~2200 m. There is no oxygen minimum at this depth, so reduction and mobilization of Mn from sediments followed by lateral transport is not a plausible source of this dMn. Further, the total Mn concentration in the deepest CB sample we analyzed (2715 m) was > 100 nM, which is higher than background levels found in other non-hydrothermally active deep basins (50 nM) (Campbell et al., 1988). These data suggest a hydrothermal input to the

deep waters of the CB. Such inputs are not entirely unexpected since it is now recognized that there are several hydrothermal activity is widespread throughout the Gulf of California region (Barragan et al., 2001; Prol-Ledesma et al., 2004; Suarez-Bosche et al., 2000). However, further work (e.g. ^{3}He, silica, Mn/Al ratios) is required to confirm the hydrothermal origin of this Mn. dMn removal rates at 2200 and 2715 m depth in CB are higher than background seawater but lower than those of the GB hydrothermal plume (Fig. 3.2C). Regardless of the origin of the elevated Mn concentrations in the deep waters of CB, the rapid Mn oxidation rates observed in the GB hydrothermal plume appear to be specific to the plume since rates either directly above the plume or at GB plume depths (~1750 – 2000 m) in CB are > 20 times slower. The known physical and chemical determinants of Mn(II) oxidation rate – temperature and oxygen concentration – are not elevated in the GB plume. Therefore, the most likely factor responsible for the rapid dMn removal rates in the GB plume is a difference in microbial activities.

Mechanism of Mn oxidation. The mechanism of microbially-mediated Mn oxidation in deep-sea hydrothermal plumes is unknown, but has been suggested to be either enzymatic (Cowen et al., 1986; Dick et al., 2006) or due to a binding mechanism involving organic polymers of microbial capsules (Cowen, 1989; Cowen et al., 1986; Cowen et al., 1990). Mn(II) oxidation has been shown to be enzymatic in bacterial isolates (Francis et al., 2001; Francis and Tebo, 2002; Rosson and Nealson, 1982) and in some studies of the upper water column of the ocean (Moffett and Ho, 1996; Tebo and Emerson, 1985). We observed a distinct temperature optimum of dMn removal at GB, suggesting an enzymatic mechanism of Mn(II) oxidation (Fig. 3.4). Temperature optimum is perhaps the most effective indicator that a geochemical process is mediated

by biology (Brock, 1978), and it has been used to demonstrate that enzymes catalyze Mn oxidation in natural samples (Tebo and Emerson, 1985; Tipping, 1984). Enzymatic activity increases with temperature to a point where activity decreases due to protein denaturation. In contrast, dMn removal by a chemical mechanism or by binding to a simple organic polymer is expected to increase proportionally with increasing temperature. It is important to note that the response of dMn removal rate to temperature shown in Fig. 3.4 likely represents the summation of activities of a mixed population of Mn(II)-oxidizing enzymes, each with a distinct temperature response. This could explain the shape of the curve, with a bell shaped optimum rather than a sharp decline in activity above the temperature optimum, which is observed for individual enzymes. For example, the relatively high dMn removal rate at 70°C could be indicative of a subset of Mn(II)-oxidizing enzymes that operate best at higher temperatures. The high temperature of the optimum dMn removal rate is also notable. Although hydrothermal plume waters are essentially the same temperature as background Gulf of California deep seawater (2.9 – 3.0°C), the temperature optimum for Mn(II) oxidation occurs at ~40°C. That the most dominant Mn(II)-oxidizing enzymes operate best at warm temperatures may indicate that the Mn(II)-oxidizing microorganisms responsible are adapted to these temperatures. One possibility is that the microbial Mn(II)-oxidizing population is endemic to warm environments near the hydrothermal source, and has been entrained in the hydrothermal plume as it rises from the seafloor. Similar mesophilic temperature responses of microbial activity have been observed in cold plume samples collected above a warm vent and black smoker at Juan de Fuca (Winn et al., 1986).

Characteristics of GB Mn(II)-oxidizing organisms and enzymes. As discussed above, the temperature optimum of dMn removal suggests that Mn(II)-oxidizing organisms in the plume could originate from warm areas near the hydrothermal source. A potential source of Mn(II)-oxidizing microbes for the plume is sediment, which can range in temperature from background (3°C) to in excess of 100°C. GB hydrothermal plumes entrain fine sediment particles as they rise from the seafloor (Campbell and Gieskes, 1984), and TEM studies have suggested that sediments are generally enriched in encapsulated or Mn-encrusted bacteria (Campbell et al., 1988; Cowen and Bruland, 1985). *Bacillus* species are common soil/sediment bacteria, and Mn(II)-oxidizing *Bacillus* have been isolated from GB hydrothermal sediments and plumes (Dick et al., 2006). These *Bacillus* species oxidize Mn(II) as spores with an enzyme (Rosson and Nealson, 1982) that resides in the outer layer of the spore (Francis et al., 2002). Several features of the dMn removal at GB are consistent with characteristics of Mn(II) oxidation by *Bacillus* spores. First, the temperature response of Mn(II) oxidation by *Bacillus* spores isolated from GB (Dick et al., 2006) and coastal sediments (Rosson and Nealson, 1982) is strikingly similar to the profile we observed in GB plume waters (Fig. 3.4). Second, dMn removal in the GB plume is resistant to heat treatment. Heat treatment (80°C for 20 minutes) is an effective method of selecting for *Bacillus* spores in environmental samples (Dick et al., 2006; Lee, 1994); spores survive this treatment whereas other organisms are killed. Heat tolerance extends to the Mn(II)-oxidizing enzyme, and has been used to select for and measure Mn(II)-oxidation by spores in the environment (Lee, 1994). In most cases, a very small fraction of dMn removal in GB samples was lost after heat treatment, consistent with catalysis by a spore enzyme (Fig.

3.5). Despite these similar properties of Mn(II) oxidation by *Bacillus* spores in culture and in natural GB hydrothermal plume waters, the dMn removal rate of the GB plume is unlikely to be attributable solely to *Bacillus* spores. Cell-specific Mn(II)-oxidation rates have been estimated at 0.0283 x 10^{-9} μM/h per spore (K. Toyoda and B.M.T., in preparation), which would require up to 2 x 10^{5} spores/ml to account for the dMn removal rates we measured in the GB plume. Maximum total cell numbers observed in the GB plume are ~ 2 x 10^{5} (Lam, 2004), and based on molecular methods, *Bacillus* species are not abundant members of the plume microbial community (G.J.D. and B.M.T., in preparation). While there are certainly limitations in the measurement of cell-specific Mn(II)-oxidation rates and in the ability of molecular methods used to describe microbial community composition, the rough calculations above suggest that there are organisms that have yet to be identified that are important catalysts of Mn(II)-oxidation in the GB hydrothermal plume.

Multicopper oxidase (MCO) enzymes are involved in Mn(II) oxidation in diverse Mn(II)-oxidizing bacteria (Brouwers et al., 1999; Corstjens et al., 1997; van Waasbergen et al., 1996) and were recently shown to be the direct catalyst of Mn(II) oxidation by marine *Bacillus* spores (G.J.D. et al., in preparation). MCOs utilize Cu as a cofactor, and in laboratory cultures Cu stimulates Mn(II)-oxidizing activity (Brouwers et al., 2000a; Larsen et al., 1999; van Waasbergen et al., 1996). We examined the effect of $CuCl_2$ additions on dMn removal rates in GB plume waters (Table 3.3). 1nM added $CuCl_2$ stimulated dMn removal by a small yet significant amount in one sample (sample 2-8 #3) but not in another (2-18 #1). A more dramatic effect of Cu was inhibition of dMn removal at higher $CuCl_2$ concentrations. At 10 nM added $CuCl_2$ (approximately equal to

the dMn concentration) dMn removal was inhibited by ~50%, and at higher concentrations inhibition approached 100%. This inhibition does not necessarily exclude MCOs as the major catalysts of Mn(II) oxidation in the GB plume. Cu addition experiments to Mn(II)-oxidizing bacterial cultures have shown a Cu optimum with inhibition of Mn(II) oxidation at higher Cu concentrations, and Cu has been suggested to compete with Mn at the Mn-binding site (van Waasbergen et al., 1996). Althought the mechanism of inhibition we observe is unknown, it is possible that the Cu additions presented here were above the optimum Cu concentration. Cu is enriched in hydrothermal fluids relative to seawater and therefore could have an important effect on dMn removal at hydrothermal vent environments. At 21°N on the East Pacific Rise, end-member Cu concentrations in hydrothermal fluids can reach 44 μmoles/kg (Von Damm et al., 1985a). In contrast, at GB and the Galapagos spreading center metal sulfides are stripped from fluids in the subsurface, and Cu concentrations are much lower (usually below detection limits but up to 1.1 μmoles/kg) (Von Damm et al., 1985b). Coincidentally, Galapagos and GB are two vent sites where Mn(II) oxidation is most rapid and Mn oxides are prevalent in plumes or as deposits. While the presence of Fe may be important in limiting microbial Mn(II) oxidation (Campbell et al., 1988; Mandernack and Tebo, 1993), our results suggest that the Cu concentration may also be important. Further studies are required to determine the effect of speciation and bioavailability of Cu, as well as how Cu inhibition of dMn removal varies across gradients of dMn concentration.

Summary and Conclusions. Microorganisms catalyze Mn(II) oxidation in the Guaymas Basin hydrothermal plume, and are responsible for dMn removal rates that are

much faster than any other deep-sea hydrothermal vent environment observed to date. This rapid microbial Mn(II) oxidation is mediated by enzymatic activity, and is specific to the hydrothermal plume and therefore unrelated to the increased flux of organic matter from productive surface waters. Plume-specific microbially-mediated Mn(II) oxidation likely reflects a unique microbial community in the hydrothermal plume. Despite early work that showed increased biomass in hydrothermal plumes (Cowen et al., 1986; Winn et al., 1986), little is known about how deep-sea microbial communities respond to the flux of potential energy sources (e.g. H_2, NH^{4+}, CH_4, H_2S, Fe^{2+}, Mn^{2+}) from hydrothermal vents. In the Guaymas Basin hydrothermal plume, concentrations of NH^{4+} and CH_4 are particularly high. Biomass, however, is only marginally higher in the plume; total cell numbers within the plume are 2-3 times that of overlying waters (Lam et al., 2005), and extracted DNA concentrations are not significantly higher than background deep seawater (G.J.D. and B.M.T., unpublished results). Thus increased biomass in the plume cannot account for the ~200 fold increase in dMn removal rates. We conclude that rapid Mn(II) oxidation in the plume is likely due to either a phylogenetically- or physiologically-distinct microbial population. Based on cell-specific Mn(II) oxidation rates of microorganisms in culture (Toyoda, in preparation), a large shift in microbial community structure would be required to account for the dMn removal rates we report in this study. Such a shift has been reported in the Suiyo Seamount hydrothermal caldera, which is dominated by just two bacterial phylotypes (Sunamura et al., 2004). Alternatively, the plume-specific rates could be the result of increased metabolic activity or differential expression of Mn(II)-oxidizing enzymes in the plume versus background deep seawater. Molecular biological studies are required to test these hypotheses and

investigate microbial community dynamics in hydrothermal plumes. Deep-sea hydrothermal vents impact ocean chemistry, and the ultimate fate of hydrothermal inputs is determined by geomicrobial transformations that occur in plumes. Our work suggests that in the case of Mn in the Guaymas Basin hydrothermal plume, these transformations are actively mediated by enzymes of distinct microbial populations.

Table 3.1. Summary of dMn Removal Rate Measurements

Sample (station-cast, #bottle)	Depth (m)	dMn (nM)	tMn (nM)	Mn oxidation rate (nM/hr)	dMn Residence time (hrs)
1-2, #3	1851	25	235	2.52	10
2-27a, #1	1950	40	288	1.70	24
1-1, #9	1800	37	247	0.43	86
1-3, #1	1900	83	178	1.96	42
2-10, #11	1890	35	159	1.44	24

Table 3.2. Control dMn Removal Experiments

Sample (station-cast, #bottle)	Depth (m)	dMn (nM)	tMn (nM)	dMn removal (% of control)			
				Azide	Anoxic	Mn rinse[a]	Mn add.[b]
1-6 #7	1963	62	315	N.D.	N.D.	85	100
1-2 #11	1900	53	130	46	38	N.D.	N.D.
1-3 #1	1912	83	178	11	27	97	N.D.
1-12 #21	1700	13	15	28	N.D.	79	N.D.
1-12 #19	1800	12	7	24	N.D.	75	N.D.
1-12 #3	1900	21	25	49	N.D.	67	N.D.
1-12 #1	1975	15	61	34	N.D.	107	N.D.
1-17 #1	1959	52	207	34	41	N.D.	N.D.

N.D. = not determined.
[a]Mn rinses were done on Mn oxides on filters, see *Materials and Methods*.
[b]Mn addition; Mn added directly to incubation bottle, see *Materials and Methods*.

Table 3.3. Effects of Copper Additions on dMn Removal

Sample	Depth (m)	dMn (nM)	tMn (nM)	dMn removal (% of control)							
				Control	azide	$CuCl_2$ Additions (nM)					
						.1	1	5	10	100	1000
2-4 #1	1762	5	138	100 (26)	15 (4)	N.D.	N.D.	N.D.	37 (14)	13 (4)	3 (0.1)
2-8 #3	1953	19	219	100 (18)	15 (1)	92 (5)	136 (2)	N.D.	47 (7)	N.D.	N.D.
2-18 #1	1898	15	211	100 (13)	N.D.	83 (19)	93 (24)	83 (6)	N.D.	N.D.	N.D.

Standard deviation of ^{54}Mn removal from triplicate incubation bottles is indicated in parentheses. N.D. = not determined.

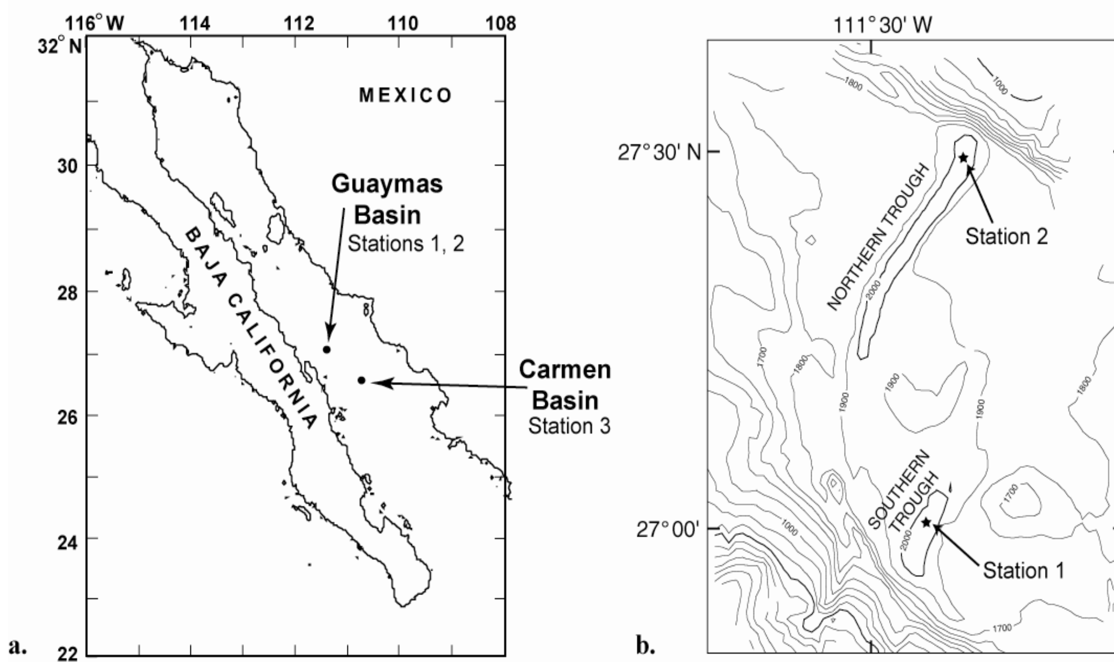

Figure 3.1. (a) Geographic location of study sites Guaymas Basin and Carmen Basin in the Gulf of California. (b) Bathymetry map of Guaymas Basin and locations of stations 1 and 2.

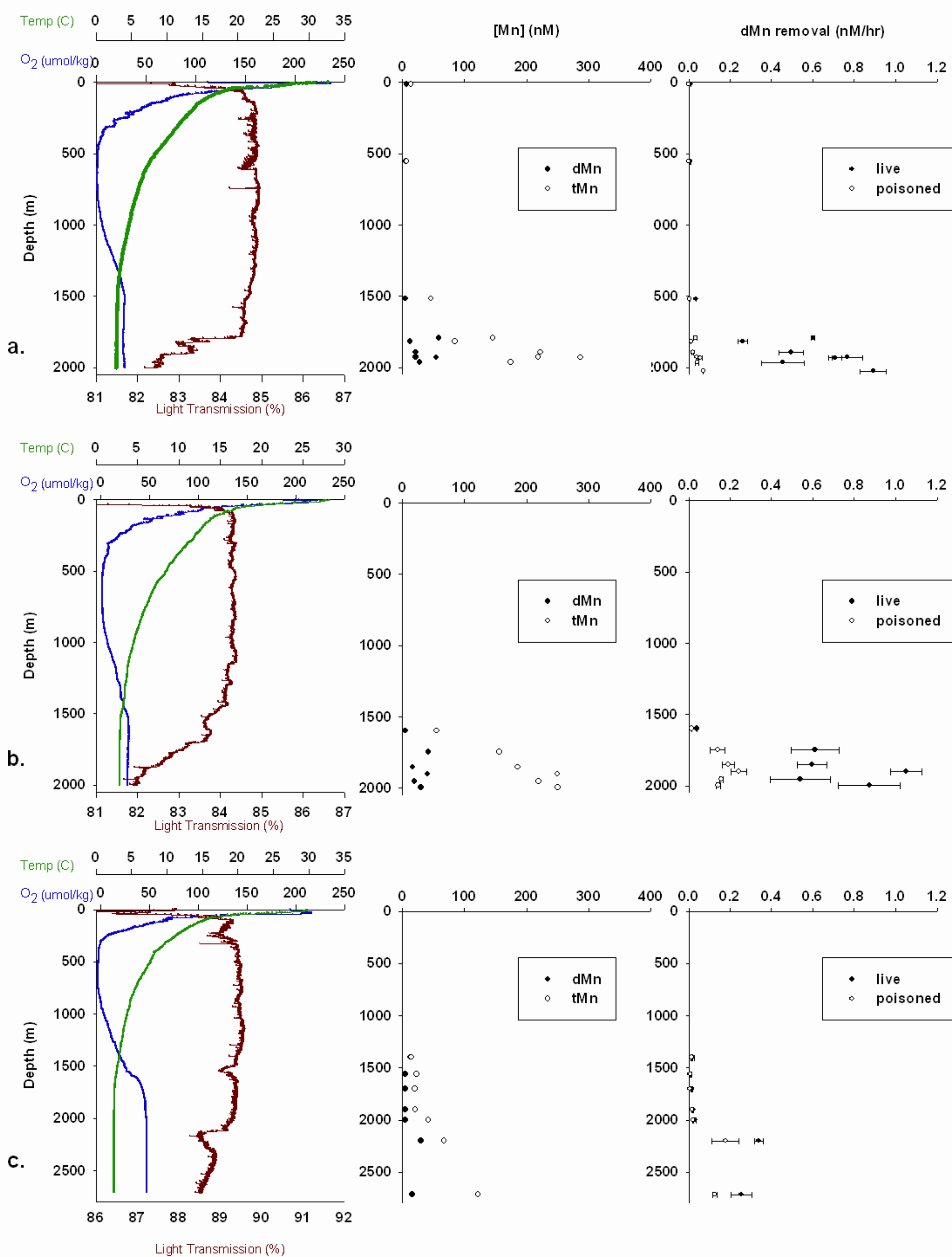

Figure 3.2. Representative depth profiles of dMn removal rates in GB southern trough (a), northern trough (b), and Carmen Basin (c). Error bars represent standard deviation of ^{54}Mn removal from triplicate incubation bottles.

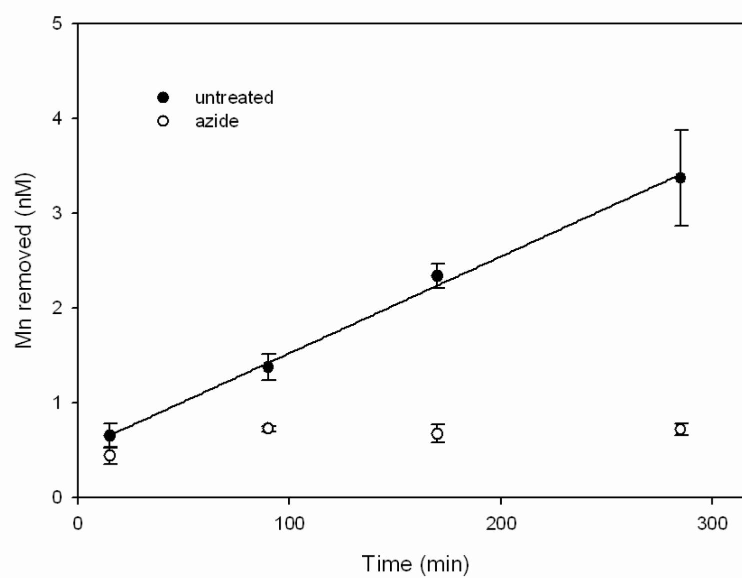

Figure 3.3. Time Course of dMn removal from sample 2-10 #11. Error bars represent standard deviation of ^{54}Mn removal from triplicate incubation bottles.

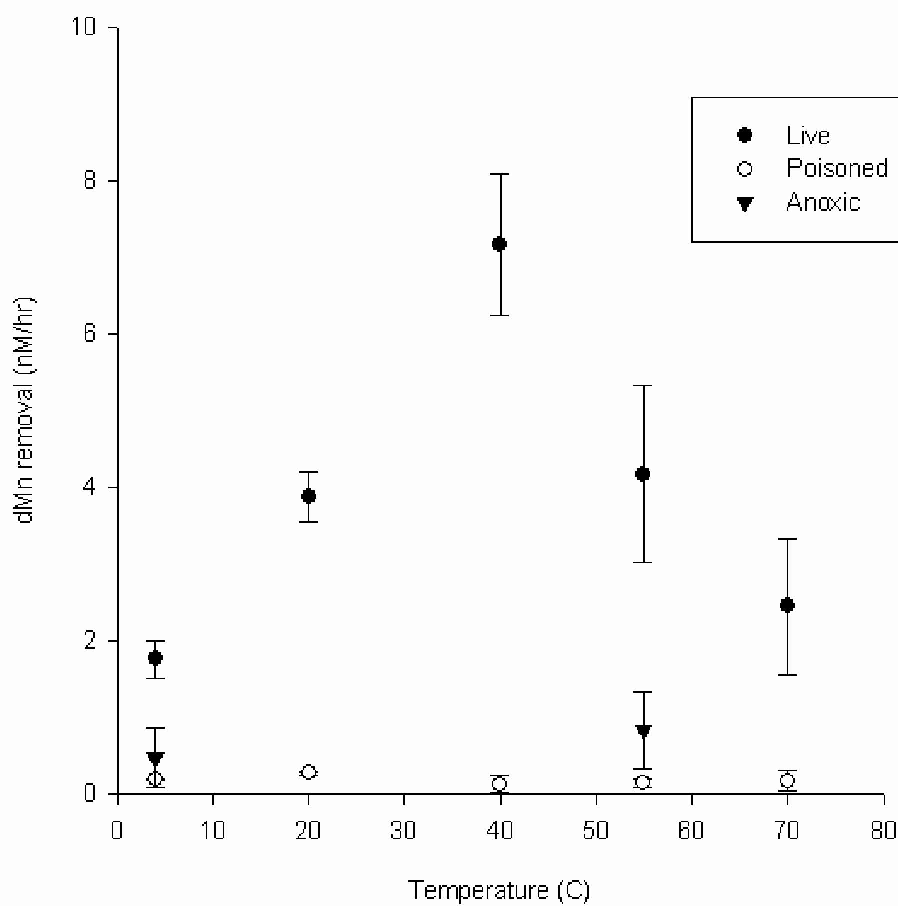

Figure 3.4. Temperature optimum of dMn removal. Error bars represent standard deviation of ^{54}Mn removal from triplicate incubation bottles.

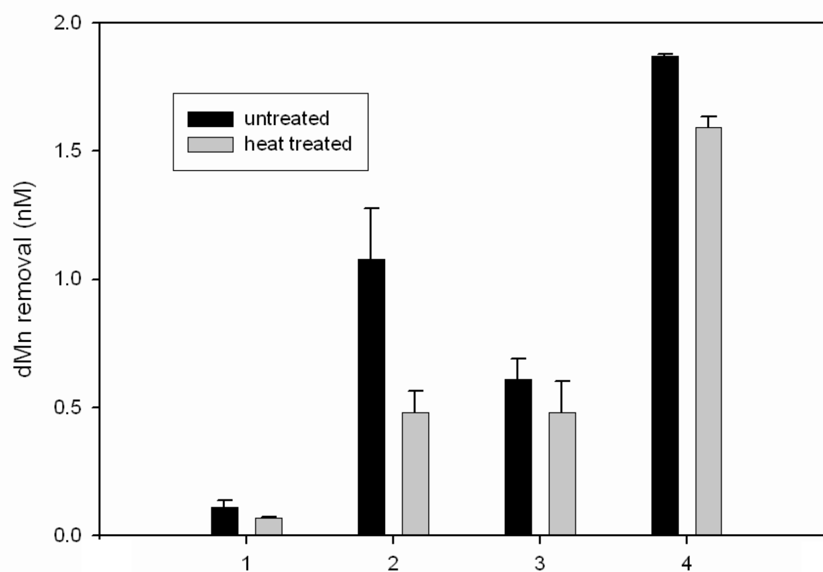

Figure 3.5. Effect of heat treatment (80°C 20 minutes) on dMn removal. Samples 1, 2, and 3 are the three samples from Table 3.3. Sample 4 is 2-27a (depth = 1950 m; dMn 40 nM; tMn 288 nM). Error bars represent standard deviation of ^{54}Mn removal from triplicate incubation bottles.

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IV

Microbial Diversity of the Guaymas Basin Hydrothermal Plume: Subtle Plume Signatures Despite Dramatic Differences in Mn(II) Oxidation Rates

Abstract

In Chapter 3, rapid Mn(II) oxidation in the Guaymas Basin (GB) hydrothermal plume was shown to be enzymatically catalyzed by a microbial community that is distinct from background deep-seawater. Here we use a molecular approach to compare microbial communities in the GB hydrothermal plume and background deep-seawater, and cultivation to identify new Mn(II)-oxidizing bacteria from the plume. Domain specific primers for eubacteria and archaea were used to construct 16S rRNA gene clone libraries from two plume samples and two background deep-seawater samples, resulting in 1,100 sequences total. All eubacterial clone libraries were dominated by γ-proteobacteria, while archaeal clone libraries were dominated by Marine Group I Crenarchaeota. Despite the dramatic difference in Mn(II) oxidation rates between the GB hydrothermal plume and background seawater, the overall microbial diversity is strikingly similar in plume and background. The biggest difference between these two types of samples was that two different clusters of sequences that are phylogenetically related to methanotrophs were overrepresented in the plume versus background. We conclude that rapid plume-specific Mn(II) oxidation rates are likely the result of plume-specific microbial activities rather than plume-specific types of microbes.

Introduction

At deep-sea hydrothermal vents, reducing fluids are injected into oxic deep-sea waters, generating a redox interface where microorganisms thrive on inorganic energy sources and sustain famously dense and diverse ecosystems. Although these near-vent

environments are conspicuous and have received much attention, a large portion of the geothermal reducing power of vents is dispersed more widely when hydrothermal fluids rapidly mix with cold seawater, forming hydrothermal plumes that rise hundreds of meters off the seafloor and travel up to thousands of kilometers from their sources. In plumes there are geomicrobial transformations of methane, ammonium, sulfur, iron and manganese that ultimately determine the fate of these hydrothermal inputs in the oceans (Lilley et al., 1995). Modern molecular methods have not been widely applied to deep-sea hydrothermal plumes, thus little is known about the microorganisms or molecular mechanisms that are responsible for biogeochemical transformations in plumes, or how deep-sea microbial communities respond to the potential energy sources that are present in hydrothermal inputs.

Guaymas Basin (GB) in the Gulf of California hosts a deep-sea hydrothermal system that is unusual because of its location in a semi-enclosed basin and its proximity to the coast. Due to productive surface waters and high sedimentation rates, the ridge axis is blanketed by a 400 m layer of sediment that chemically modifies hydrothermal fluids as they ascend towards the seafloor (Von Damm et al., 1985b). In particular, hydrothermal fluids are enriched with ammonium, methane, and hydrocarbons, whereas ore-forming metals such and iron and copper are precipitated and removed. Mn remains in solution, resulting in hydrothermal fluids with unusually high Mn/Fe ratios. These fluids are then injected into a semi-enclosed basin, where hydrothermal inputs are trapped and accumulate to much higher concentrations than at mid ocean ridges. Mn is especially prominent in the GB hydrothermal plume; dissolved Mn(II) is oxidized and precipitated as Mn oxide minerals, forming turbid hydrothermal clouds that sit in the basin (Campbell

et al., 1988). Mn oxides in these clouds are dominated by particles that resemble Mn oxide-encrusted microbes (Campbell et al., 1988), and indeed rapid Mn(II) oxidation rates are enzymatically catalyzed by microorganisms (Chapter 3). Rapid Mn(II) oxidation rates are specific to the GB plume, being 20 to 200 times faster than background deep-seawater either just above the plume or at plume depths in a neighboring basin, Carmen Basin, which is not impacted by hydrothermal activity at these depths (Chapter 3). The overall chemistry of background and plume waters is very similar, as are the concentrations of known chemical/physical determinants of Mn(II) oxidation rates determinants, O_2 and temperature. Therefore rapid plume-specific Mn(II) oxidation rates likely reflect a hydrothermal plume microbial community that is distinct from that of background deep-seawater, making GB a perfect natural laboratory to investigate microbial populations involved in Mn(II) oxidation and other geomicrobial transformations as they occur in the environment.

A recent study analyzed specific populations of methane and ammonia-oxidizing bacteria in the GB plume (Lam, 2004), but nothing is known about the overall microbial diversity of the GB plume or the microbes that are responsible for rapid Mn(II) oxidation rates. Several considerations suggest that the GB hydrothermal plume is composed of unique types of microbes that are not present in background deep-seawater. First, hydrothermal plumes are enriched in potential substrates for chemolithoautorophic growth (especially methane and ammonium), whereas energy resources are scarce in the deep-sea. Second, although total cell numbers in the plume are elevated up to two times those of overlying waters (Lam, 2004), this increase is not nearly high enough to account for the difference in observed Mn(II) oxidation rates. Third, based on cell-specific

Mn(II) oxidation rates of bacteria in culture, a large fraction of cells present in the GB plume are required to account for the observed GB plume Mn(II) oxidation rates (Chapter 3). Therefore, we hypothesized that the most abundant members of the GB plume microbial community are phylogenetically distinct from those of background deep-seawater. Here we analyze the microbial diversity of the GB plume and compare it to background Gulf of California deep-seawater. Genetic probes specific for Mn(II)-oxidizing bacteria or their functional genes are not yet available, therefore we used a PCR-based approach with primers specific to bacteria or archaea to analyze overall microbial diversity, and cultivation to identify new Mn(II)-oxidizing bacteria. Our results reveal that despite dramatic differences in Mn(II) oxidation rates in plume and background seawater, differences in the overall microbial community are subtle. We suggest that the unique biogeochemical transformations that occur in the GB hydrothermal plume are the result of plume-specific activities of endogenous deep-sea microbial populations.

Materials and Methods

Sample collection and cultivation of Mn(II) oxidizers. Sampling and procedures for cultivation were as described in Table 4.3 and Chapter 2. The study site and plume sampling techniques were as described in Chapter 3. Samples for clone libraries are described in Table 4.1; water was collected in 10 L Niskin bottles, and filtered directly from the bottle onto a 142 mm 0.2 µM polycarbonate filter membrane by N_2 gas pressure filtration at < 5 psi. Filters were treated with RNase away (Ambion) as

recommended by the manufacturer, incubated at 4°C overnight then stored at -20°C for the duration of the cruise and at -80°C upon return to the shore lab.

DNA extraction. DNA was extracted from ¼ of filter per tube. The ¼ filter was cut into small pieces (~1 x .25 cm) and added to a 1.5 ml screw-top centrifuge tube containing 200 mg each of 0.1, 0.5 and 2 mm zirconium beads. The following were then added: 580 µl lysis solution (300 mM EDTA, 300 mM NaCl, 300 mM Tris buffer, pH 7.5), 70 µl 15% SDS, and 35 µl 1M DTT in 0.01 M Na acetate. The tubes were vortexed, incubated at 70°C for 30 minutes, and cooled to < 40°C. 14 µl 5% lysozyme (w/v in water) was added and the tubes were incubated at 37°C for 20 minutes then beat on a FastPrep bead beater machine for 45 seconds at setting 6.5. 150 µl 1M KCl was added, then the tubes were vortexed and placed on ice for 2-5 minutes. The mixture was then centrifuged for 5 minutes at 14,000 x *g*, the clear supernatant was transferred to a Montage PCR purification filter unit, and it was centrifuged at 1,000 x *g* until all of the liquid passed through (~15 minutes). The flow-through was discarded, and 400 µl TE buffer was added to the filter unit and it was centrifuged for 15 minutes at 1,000 x *g*. The DNA was then eluted in 100 µl TE as described by the manufacturer. DNA was quantified using PicoGreen (Invitrogen).

PCR, cloning, and sequencing. Eubacterial 16S rRNA gene PCR was done with primers 27f and 1492r as modified by (Vergin et al., 1998) and 21f and 958r were used for archaea (Huber et al., 2002). The PCR reaction contained 1X reaction buffer (Roche), 0.2 mM dNTPs, 0.1 µM each primer, 5 ng template DNA, and 0.5 U Taq (Roche). As few PCR cycles as possible were used in an effort to minimize PCR artifacts (Acinas et

al., 2005). 8 replicate 25 µl PCR reactions were run for one cycle of 94°C for 3 minutes, twenty cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes (for archaeal PCR, 25 cycles were required), and 1 cycle of 72°C for 8 minutes. The replicates were then combined and cleaned with the Qiagen PCR purification kit, and eluted in 40 µl water. 2.5 µl of this product was then diluted 10x in fresh PCR mixture, and "reconditioned" under the above conditions for 5 cycles (Acinas et al., 2005). It was then purified with the Qiagen PCR purification kit, and 4 µl was used in a TOPO cloning reaction with pCR2.1 as described by the manufacturer (Invitrogen). White colonies were picked, grown, harvested, and plasmid DNA extracted using the DirectPrep 96 miniprep kit (Qiagen). Both strands of the 5' end of the eubacterial 16S rRNA gene was sequenced with primer 536r and M13f/r. For archaea clones, the entire insert was sequenced with M13f/r.

Clone library and phylogenetic analysis. DNA sequences were edited and assembled with Sequencher (GeneCodes). Sequences were oriented and checked for chimeras with OrientationChecker and Mallard (Ashelford et al., 2006), respectively (http://www.cf.ac.uk/biosi/research/bioft/index.html). Eubacterial sequences were classified using the RDP Classifier (http://rdp.cme.msu.edu/index.jsp). Sequences were aligned and edited manually using MEGA3.1 (Kumar et al., 2004). Distances matrices generated with MEGA3.1 (Jukes-Cantor evolutionary model, default settings) were used for cluster analysis and diversity statistics using DOTUR (Schloss and Handelsman, 2005). Phylogenetic analysis was done by neighbor-joining within MEGA3.1 using the Kimura 2 parameter evolutionary model and 1,000 bootstrap replicates.

Results and Discussion

DNA for 16S rRNA gene clone libraries was extracted from two GB hydrothermal plume samples and two background deep-seawater samples (Table 4.1). Because hydrothermal venting influences the entire GB below its sill depth (1560 m), no suitable background controls are available from within the basin. Therefore we used a sample from just above the plume (A) and one from the same depth as the GB plume in a neighboring basin, Carmen, which is not influenced by hydrothermal activity (B). Mn(II) oxidation rates were much higher in the plume versus background (Table 4.1). Partial 16S rRNA sequences from a total of 1,106 clones were analyzed, and coverage estimates indicate that the most abundant community members were sampled in all libraries (Table 4.2). Chao1 (Chao, 1984; Chao et al., 1993) and Shannon (Shannon and Weaver, 1949) diversity indices clearly showed that the eubacterial communities are much more diverse than the archaea, however there was no significant difference in diversity between plume and background (Table 4.2). All eubacterial clone libraries were dominated by proteobacteria, especially γ- and α-proteobacteria (Fig. 4.1). At the phylum level, there were no discernable differences between plume and background samples (Fig. 4.1).

All sequences were grouped into operational taxonomic units (OTUs) that share 97% sequence identity (Fig. 4.2). For the eubacteria, 17 OTUs contained a total of 10 or more sequences, and in no case were there sequences found in plume but not background samples (the top 8 are shown in Fig. 4.2A). Two eubacterial OTUs were consistently overrepresented in plume samples relative to background. OTU 3 sequences were more

than twice as abundant in plumes clone libraries than background (Fig. 4.2A); BLAST analysis indicates that these sequences are most closely related to uncultured γ-proteobacteria from methane-rich environments and cultured methylotrophs of the genus *Methylomonas*. OTU 7 sequences are β-proteobacteria that were greater than three times more abundant in the plume versus background (Fig. 4.2A). Clustering analysis of archaeal sequences revealed extremely low diversity in the GB plume; 295 out of 411 total sequences fit into a single OTU (1) that contains members of the Marine Group I Crenarchaeota (Fig. 4.2B). As with the eubacteria, all archaeal sequences that were abundant in the plume are also present in background deep-seawater, indicating that none of the most abundant plume clones are specific to the plume at the 97% 16S rRNA gene level.

Phylogenetic analysis was performed on representatives of the most abundant and diverse sequences (Fig. 4.3). Clone 1E-159 (OTU 3) represents the single most abundant eubacterial sequence (49 clones) and clustered with uncultured sequences retrieved from a mud volcano (Loesekann et al., unpublished) and a methane-rich borehole (Carson et al., 2003). These sequences are also closely related to *Methylomonas methanica*, a methanotroph from a lake sediment (Costello and Lidstrom, 1999). OTU 3 clones were highly overrepresented in plumes (37 clones) versus background (12 clones), suggesting that this group of organisms is stimulated by elevated methane present in the plume. Methane concentrations in buoyant plumes and neutrally-buoyant plumes can reach up to 11.2 and 31.6 µM, respectively, compared to just 16-65 nM in the overlying water (Lam, 2004). Two other abundant OTUs (1, 4) were also composed of γ-proteobacteria that cluster phylogenetically with methanotrophs and/or thiotrophs. OTU 1, represented by

clones 3E-087 and 1E-155, contains sequences that are most closely related to chemoautotrophic symbionts of *Bathymodiolus* mussels (Distel et al., 1988; Duperron et al., 2006; McKiness et al., 2005) and *Vesicomya* clams (Peek et al., 1998) that inhabit deep-sea vent environments, as well as uncultured organisms from the Suiyo Seamount (Sunamura et al., 2004) and diffuse flow at Axial Volcano, Juan de Fuca Ridge (Huber et al., 2003) (Fig. 4.3A). OTU 4 sequences, represented by clones 1E-188 and 1E-143, were most similar to sequences from uncultured organisms of deep-sea plumes and background deep-seawater (Huber et al., 2003). This group also clusters with a number of clones (1E-7, 2E-55) that are very closely related to methanotrophic gill symbionts (Duperron et al., 2006). All sequences within OTUs 1 and 4 were represented equally in plume and background clone libraries, indicating that these groups are abundant as free-living organisms in deep-seawater in addition to hydrothermal and/or animal host-associated environments.

Most Mn(II)-oxidizing isolates were also identified as γ-proteobacteria. Five of the isolates (GB02-2B, -2C, -3, -4, and -19) were retrieved from warm microbial mats in hydrothermal sediments (Table 4.3) and cluster with *Microbulbifer* and/or *Pseudomonas* type organisms, some of which are close to known Mn(II) oxidizers (Templeton et al., 2005). Genetic distance from described bacteria (97% 16S rRNA) suggests that in some cases these isolates likely represent new species of bacteria, and their identification as Mn(II) oxidizers expands the known diversity of organisms able to catalyze this process. Another Mn(II)-oxidizing isolate was from cold sediment and was identical by 16S rRNA to a known Mn(II)-oxidizer, *Photobacterium profundum* strain 3TCK (C.A. Francis et al., unpublished). In chapter 3, we suggested that the mesophilic temperature optimum

(~40°C) of Mn(II) oxidation rates in the GB plume could be the result of mesophilic organisms present in warm sediments (such as those described here) that are entrained into hydrothermal fluids as they rise from sediments into the plume. However, the absence of these sequences or any other abundant GB sediment sequences (Teske et al., 2002) in our GB plume clone libraries suggests there is not a strong sediment signature in the plume microbial community. One Mn(II)-oxidizer that was isolated from the GB plume, GB02-35C, clusters with *Pseudoalteromonas* species and is identical to one clone and similar to two others that were retrieved from the GB plume (Fig. 4.3A). Several Mn(II)-oxidizing *Pseudoalteromonas* species have also been isolated from an area of intense Mn(II) oxidation in the suboxic zone of the Black Sea, where they also appear to be abundant based on molecular community analysis (B.G. Clement thesis). In contrast, only a few Pseudoalteromonas sequences were present in the GB plume clone libraries, so they do not appear to be among the most abundant community members of the plume.

Abundant α-proteobacteria sequences in GB plume clone libraries were generally most similar to sequences from uncultured organisms that are common in seawater. OTU 5, represented by clones 2E-25, 1E-17, 1E-29, and 1E-179, is most similar to sequences retrieved from diffuse flows at Axial Volcano, JDF(Huber et al., 2003), and also clusters with *Pelagibacter ubique*, a cultured representative of the abundant SAR11 cluster of marine bacteria (Morris et al., 2002) (Fig. 4.3B). OTU 6 sequences (4E-079, 2E-136) clustered with sequences from background deep-seawater (Huber et al., 2003) and ridge flank crustal fluids (Huber et al., 2006) (Fig. 4.3B). While several clones from OTUs 5 and 6 clones (1E-179, 1E-17, 4E-079) appear to be overrepresented in the GB plume versus background, the sampling size is small and therefore of questionable significance.

Targeted FISH probes should be applied to further assess the distribution of these organisms in the deep-sea and in hydrothermal plumes. One Mn(II) oxidizing isolate (GB02-44A, Table 4.3) and a number of clone library sequences were scattered thought the α-proteobacteria, including the *Sulfitobacter*, *Roseobacter*, and *Methylarcula* groups which harbor known Mn(II) oxidizers (Fig 4.3B). One clone (1E-75) was very closely related (622 of the 625 bases considered are identical) to the Mn(II)-oxidizing *Erythrobacter* sp. strain SD-21 (Francis et al., 2001). However, most these sequences were not well represented in clone libraries, and the α-proteobacteria are notoriously polyphyletic for the ability to oxidize Mn(II) (Chapter 6). Thus it is difficult to draw conclusions regarding the significance of these organisms in catalyzing Mn(II) oxidation in the GB plume.

The second most abundant eubacterial OTU (OTU 2, clones 4E-051, 3E-015) is comprised of sequences that are classified as either unclassified proteobacteria or δ-proteobacteria. These sequences were present in both plume and background and cluster with uncultured clone sequences from waters near Hawaii (DeLong et al., 2006) and in the Arctic Ocean (Bano and Hollibaugh, 2002) (Fig. 4.3C). ε-proteobacteria are abundant in deep-sea hydrothermal vent environments (Corre et al., 2001; Haddad et al., 1995; Longnecker and Reysenbach, 2001; Moyer et al., 1995; Reysenbach et al., 2000), however only a total of three ε-related sequences were retrieved from all four eubacterial clone libraries, reinforcing the notion that the plume community is distinct from that of hydrothermal fluids or the near-vent environment (Fig. 4.3C). Finally, eubacterial OTU 7 contains a tight cluster of sequences that fall within the β-proteobacteria and are most

similar (98%) to sequences recovered from an estuarine sediment (Nielsen et al., 2004) (Fig. 4.3C). The closest isolate to this group is *Methylotenera mobila* (94% identity), a novel, obligate methylamine utilizer (M.G. Kalyuzhnaya et al., unpublished; Genbank accession DQ287786). 14 sequences from this OTU were retrieved from plume samples versus only four sequences from background samples, making it the OTU that is most highly enriched in the plume relative to background.

Phylogenetic analysis of archaeal clone library sequences revealed that the vast majority fell within a narrow clade of the Marine Crenarchaeota Group I (MCGI) (Fig. 4.3D). Sequences from within this clade have been recovered from diverse environments such as seafloor basalts, coastal waters (Delong, 1992), Antarctic sponges (Webster et al., 2004), and deep-sea hydrothermal vents (Takai and Horikoshi, 1999). These sequences are also similar (97% identity over 838 bp) to the 16S rRNA gene for *Candidatus Nitrosopumilis maritimus*, which was recently isolated as an ammonia oxidizer (Konneke et al., 2005). The extensive diversity of ammonia-oxidizing genes (*amoA*) among the archaea suggests that ammonia oxidation may be widespread among the crenarchaeota (Francis et al., 2005). Thus the sequences we present here may represent crenarchaeota that are actively oxidizing ammonia, which is present in the plume at concentrations of up to 3 μM compared to 0.16 μM above-sill depths (Lam, 2004). However, crenarchaeota clones accounted for equal proportions of the archaea in our plume libraries as in those constructed from background seawater (Fig. 4.3D). If ammonium does stimulate crenarchaeota in the plume, we are unable to detect the stimulation based on our comparative clone library analysis. To our knowledge, no direct measurements of total archaeal cell numbers in the GB plume have been made, so it could be that all

archaea are stimulated so that the proportions of each group do not change. Regardless of whether the cell numbers of the MCGI are enhanced, it seems likely that the elevated ammonium concentrations within the plume could affect the physiological state of these organisms. Such plume-specific physiological states or metabolic activities could include Mn(II) oxidation. Because they are so abundant in the GB plume, the cultured representative of the MCGI should be tested for the ability to oxidize Mn(II).

Overall, our results indicate that although biogeochemical parameters such as concentration and rates of Mn (Chapter 3), ammonium, and methane (Lam, 2004) are greatly elevated in the GB hydrothermal plume, the overall diversity of the microbial communities in the plume is remarkably similar to that in background deep-seawater. Mn(II) oxidation rates in particular are much higher in the plume than background, and based on cell-specific Mn(II) oxidation rates of cultivated organisms, a large fraction of the cells present in the GB plume are required to account for the observed Mn(II) oxidation rates. The biggest difference we observed in plume and background clone libraries was in the abundance of clones that are most similar to methanotrophs from within the γ- and β-proteobacteria. Although these two OTUs (3 and 7) are abundant, accounting for 10.1 – 11.1 % and 2.9 – 4.8 % of the total clones in the plume samples, respectively, the increased abundance (a factor of 2 – 3) is still nowhere near high enough to account for the difference in Mn(II) oxidation rates (a factor of 20 – 200). While there are well- known biases involved in the PCR we used to generate our clone libraries (Wintzingerode et al., 1997), we feel our results are significant for the following reasons: (1) our PCR was done at a low number of cycles, well before reaching the plateau of product that might equalize samples with different quantities of starting templates; (2) our

analysis is based on comparison of samples that should have the same biases; (3) the trends we report are consistent for two samples each for plumes and background.

There are several possible explanations for similar microbial communities despite dramatic differences in Mn(II) oxidation rates. First, there could be undetected, unidentified Mn(II) oxidizers within the plume that oxidize Mn(II) many times faster than any known organisms. Second, there could be dramatic shifts in strain-level population structure between background and plume that cannot be detected at the phylogenetic resolution of the 16S rRNA gene. Third, the elevated Mn(II) oxidation rates in the GB plume could be the result of distinct activities and/or metabolic state of plume microbes rather than distinct types of microbes. While we cannot rule out the first two possibilities, there is no data to support them at this time. The third possibility seems most likely based on the fact that known Mn(II) oxidizers often oxidize Mn only under certain physiological states, for example during growth on some carbon or energy sources, but not others (see Chapter 6). The presence of elevated methane and ammonium are the two most obvious plume characteristics that might stimulate Mn(II) oxidation rates. At least some OTUs that are phylogenetically affiliated with methanotrophs are consistently elevated in the plume, suggesting that methane does stimulate some plume microbes. However, to properly assess differences in microbial metabolism between the plume and background deep-seawater, RNA and protein-based studies are required. Our results also highlight the need for the continued development of probes for functional Mn(II) oxidation genes, which requires advances in understanding of the biochemistry and molecular genetics of Mn(II) oxidation.

Table 4.1. Sample summary[a]

Sample	Lat/Long	Depth (m)	O_2 (μmol/kg)	T (°C)	tMn (nM)	dMn (nM)	Mn ox. Rate (nM/hr)
Plume A	27° 1.50'N 111° 25.07'W	1996	27.38	2.96	356	145	10.00[b]
Plume B	27° 1.85'N 111° 24.00'W	1775	27.04	2.95	257	59	0.600
Background A	27° 0.792'N 111° 25.25'W	1503	28.34	3.08	46	5	0.035
Background B	26° 22.81'N 110° 43.77'W	2000	46.49	2.57	42	5	0.030

[a]data from Chapter 3.
[b]this rate experiment was done at room temperature rather than 4°C as the others. Temperature response curves predict that the rate at 4°C for this sample would be 5 nM/hr.

Table 4.2. Clone library summary

Library		No. clones	No. unique OTUs[a]	Cover.[b]	Chao1 (95% lci – hci)[c]	Shannon (95% lci - hci)[c]
Eubacteria	Combined	693	163	86.3	357.13 (272.434-507.38)	4.08 (3.97-4.29)
	Plume A	171	58	78.9	136.75 (89.9-252.25)	3.47 (3.30-3.65)
	Plume B	189	68	76.2	178.00 (115.75-321.418)	3.57 (3.39-3.75)
	Back. A	175	72	71.4	208.11 (132.37-378.89)	3.67 (3.47-3.85)
	Back. B	159	61	79.2	101.615 (77.93-158.45)	3.64 (3.47-3.81)
Archaea	Combined	413	21	98.5	26.00 (21.87-49.91)	1.30 (1.14-1.45)
	Plume A	100	7	99.0	7.00 (n/a)	0.74 (0.50-0.99)
	Plume B	100	12	96.9	13.00 (12.09-22.68)	1.66 (1.41-1.91)
	Back. A	117	12	95.0	14.50 (12.37-28.98)	1.23 (0.96-1.51)
	Back. B	97	10	97.4	11.50 (10.15-25.08)	1.48 (1.28-1.68)

[a]Operational taxonomic units (OTUs) defined at 97% sequence identity.
[b]Coverage as calculated by the method of Good (Good, 1953); the percentage of sequences that clustered into OTUs of two or more sequences.
[c]95% lci-hci is the range of each index at 95% confidence intervals.

Table 4.3. Mn(II)-oxidizing isolates summary

Isolate	Depth (m), Temp (°C)[b]	Sample & Media	Closest Relative	% Similarity (bp considered)	Reference
GB02-2B/2C	2000, 4-31	Sediment microbial mat, K media	*Microbulbifer salidpaludis*	97 (1475)	(Yoon et al., 2003)
GB02-3	2000, 4-31	Sediment microbial mat M media	*Microbulbifer* sp. KBB-1	99 (1459)	(Templeton et al., 2005)
GB02-4	2000, 4-31	Sediment microbial mat, M media	*Microbulbifer maritimus*	96 (1446)	(Yoon et al., 2004)
GB02-19	2000, 24-74	Sediment microbial mat, K media	*Microbulbifer* sp. KBB-1	99 (1442)	(Templeton et al., 2005)
GB02-42B[a]	2000, 3.0-3.7	Sediment, M media	*Photobacterium profundum* strain 3TCK	99 (1414)	F. Lauro et al. unpublished
GB02-44A[a]	1865, 3	Plume, M	*Oceanicola batsensis* HTCC 2597	96 (1360)	(Cho and Giovannoni, 2004)

[a]these two isolates were lost upon storage.
[b]the temperature range shown indicates the range of temperature measured while holding the temperature probe steady in place, and indicates the dynamic, variable nature of the microbial mats at the hydrothermal sediment/water interface.

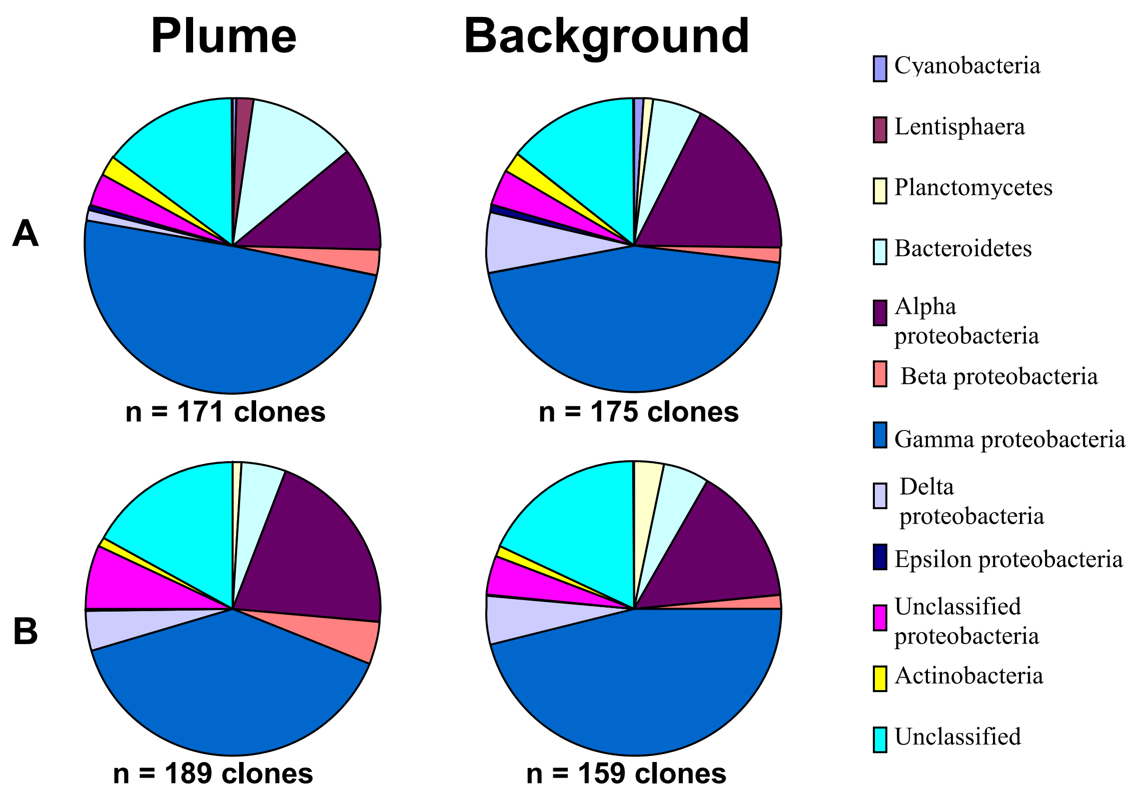
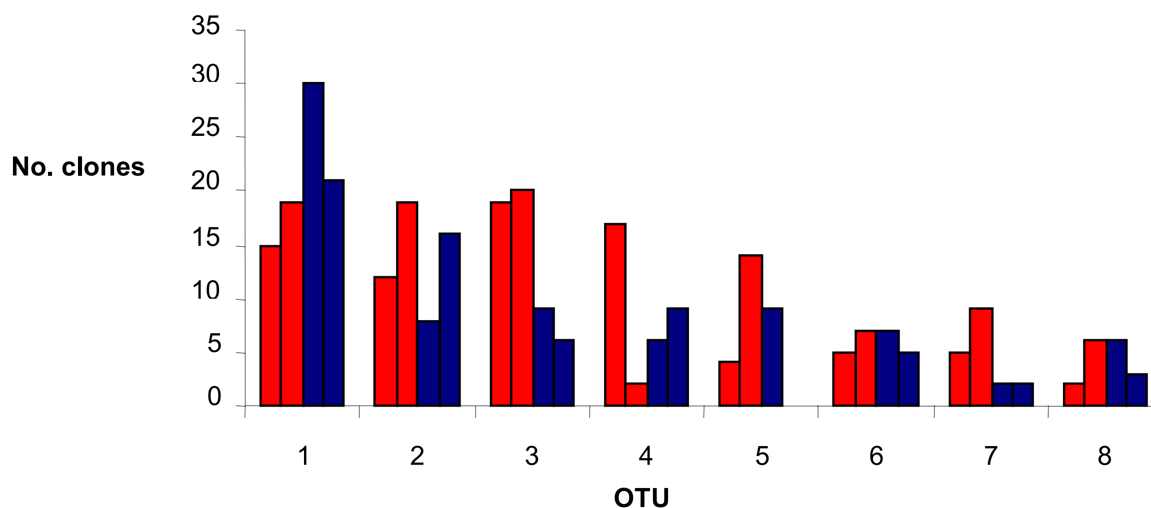
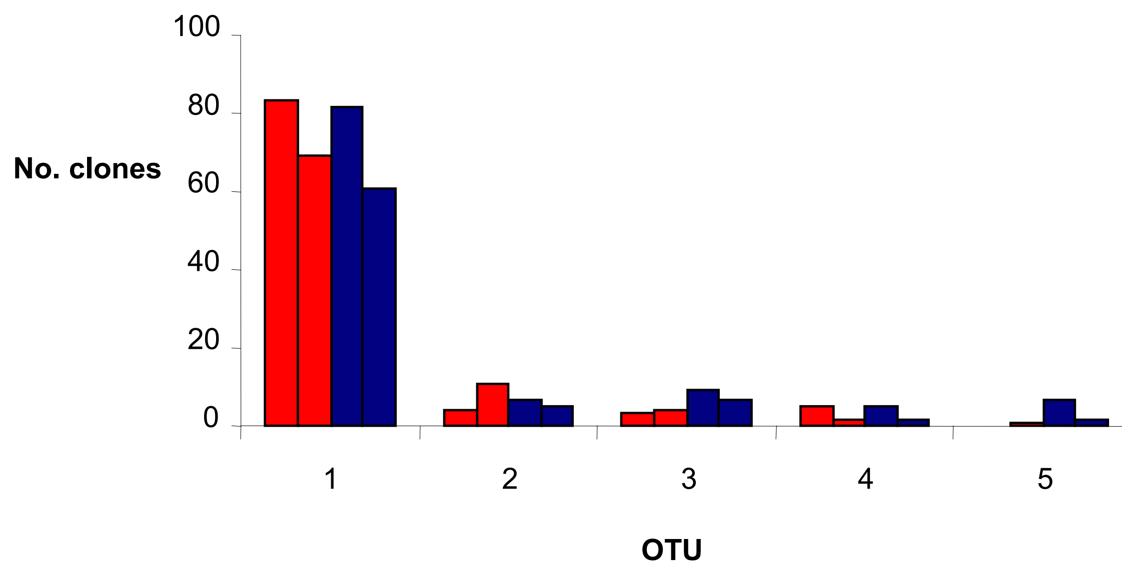


Figure 4.1. Eubacterial community composition in the GB hydrothermal plume versus background deep-seawater samples. Sequences were assigned to phylum/class with the RDP classifier.



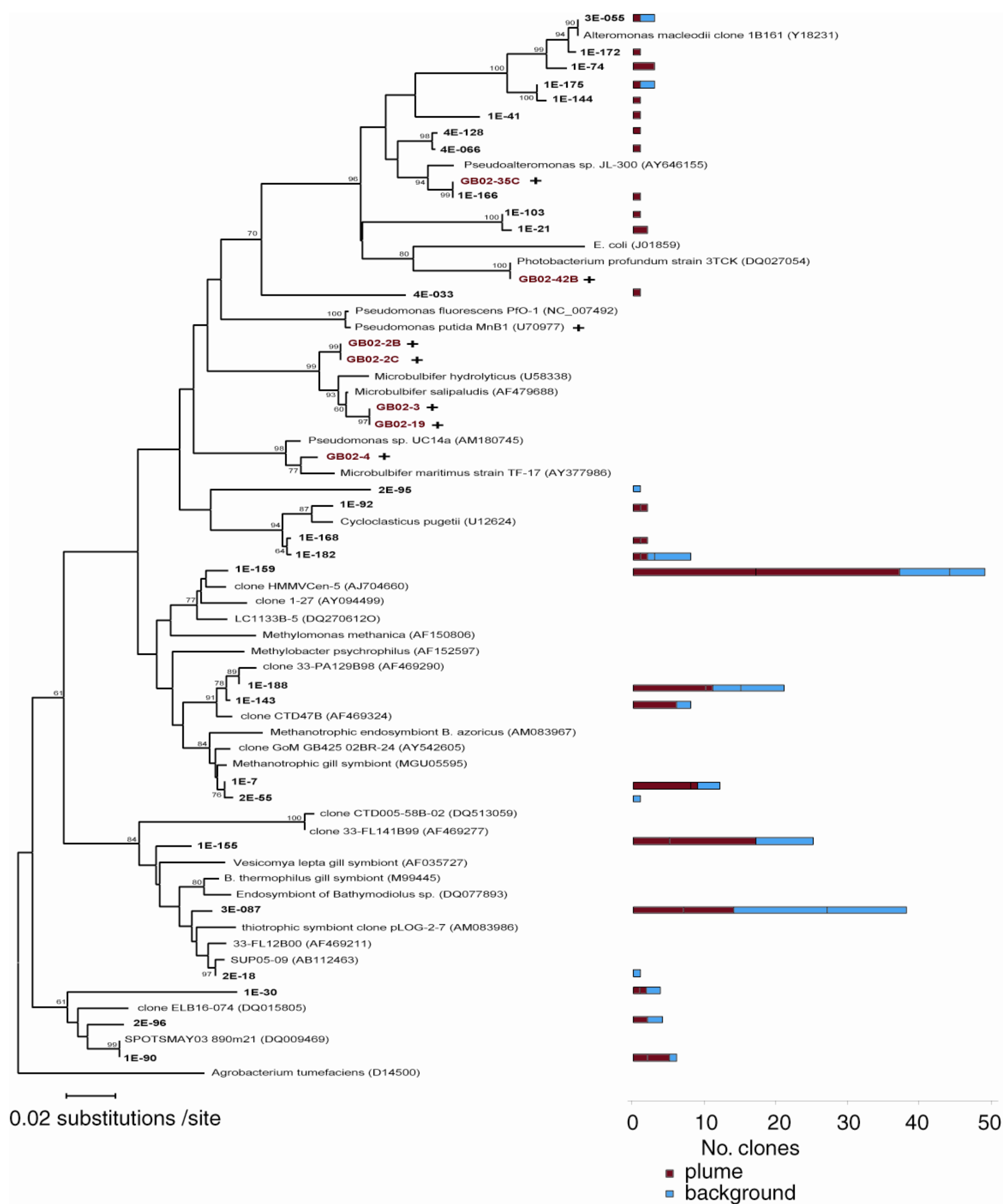
A.

Figure 4.2. Most abundant OTUs. Red columns each represent a plume sample, blue columns background deep-seawater samples. **(A)** Eubacterial OTUs. Identity of each OTU is as follows, with representative clone identifiers as shown in Fig. 4.3: (1) uncultured γ-proteobacteria mussel symbionts (3E-087); (2) uncultured unclassified/δ-proteobacteria (4E-051); (3) *Methylomonas*-like γ-proteobacteria (1E-159); (4) *Methylobacter*-like γ-proteobacteria (1E-188); (5) SAR11 cluster α-proteobacteria (1E-179); (6) uncultured SAR11-like α-proteobacteria (4E-79); (7) *Methylotenera*-like β-proteobacteria (3E-98); (8) uncultured δ-proteobacteria (1E-20, not shown on tree).



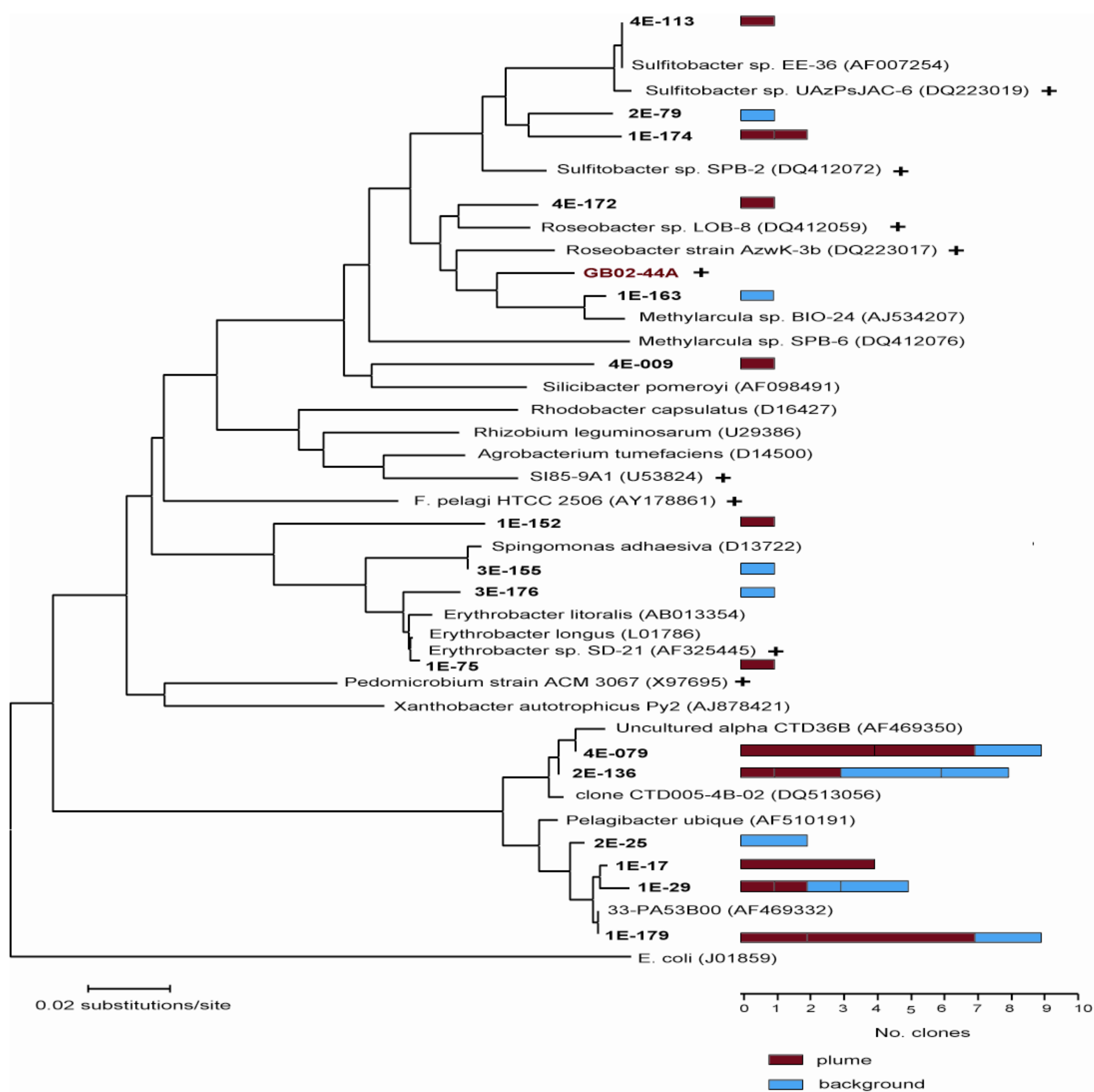
B.

Figure 4.2, Continued (B). Most abundant archaeal OTUs. Red columns each represent a plume sample, blue columns background deep-seawater samples. Identities of each OTU are as follows: (1), (2), and (5), Marine Crenarchaeota Group I; (3) and (4), Marine Euryarchaeota Group II.



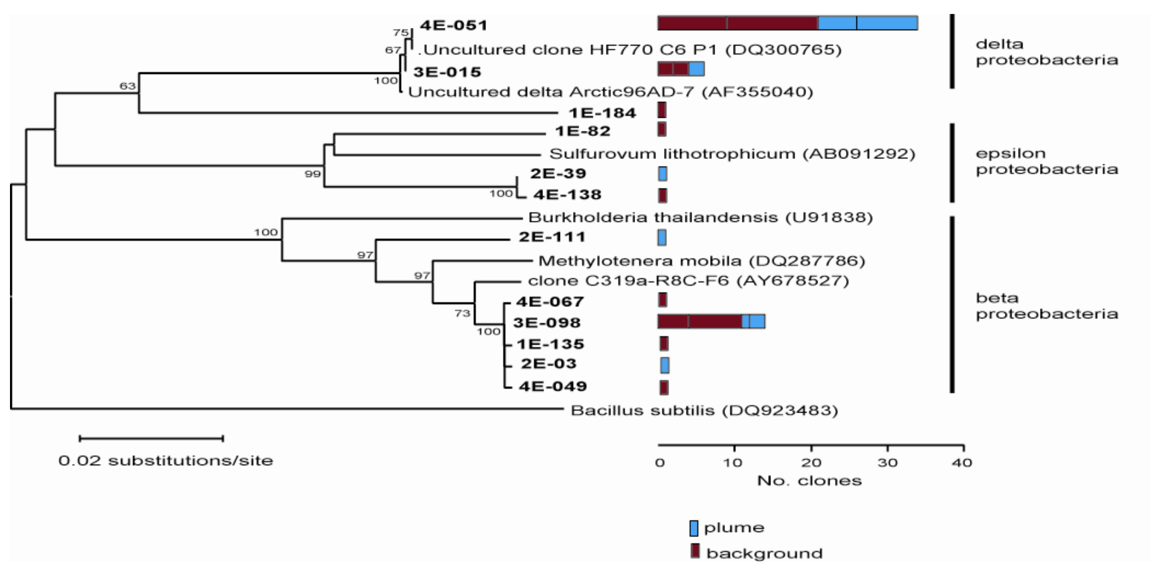
A.

Figure 4.3. Phylogenetic trees based on 16S rRNA genes of Mn(II)-oxidizing isolates and most abundant/diverse clones. Bootstrap numbers are based on 1000 replicates; only values greater than 60 are shown. Clones are shown in bold, and the number of clones retrieved for each sequence (100% identical) is indicated by the bar; red refers to sequences retrieved from the plume, blue from background deep-seawater. Mn(II)-oxidizing isolates are indicated with "+", the names of those from GB are shown in red. **(A)** gamma proteobacteria.



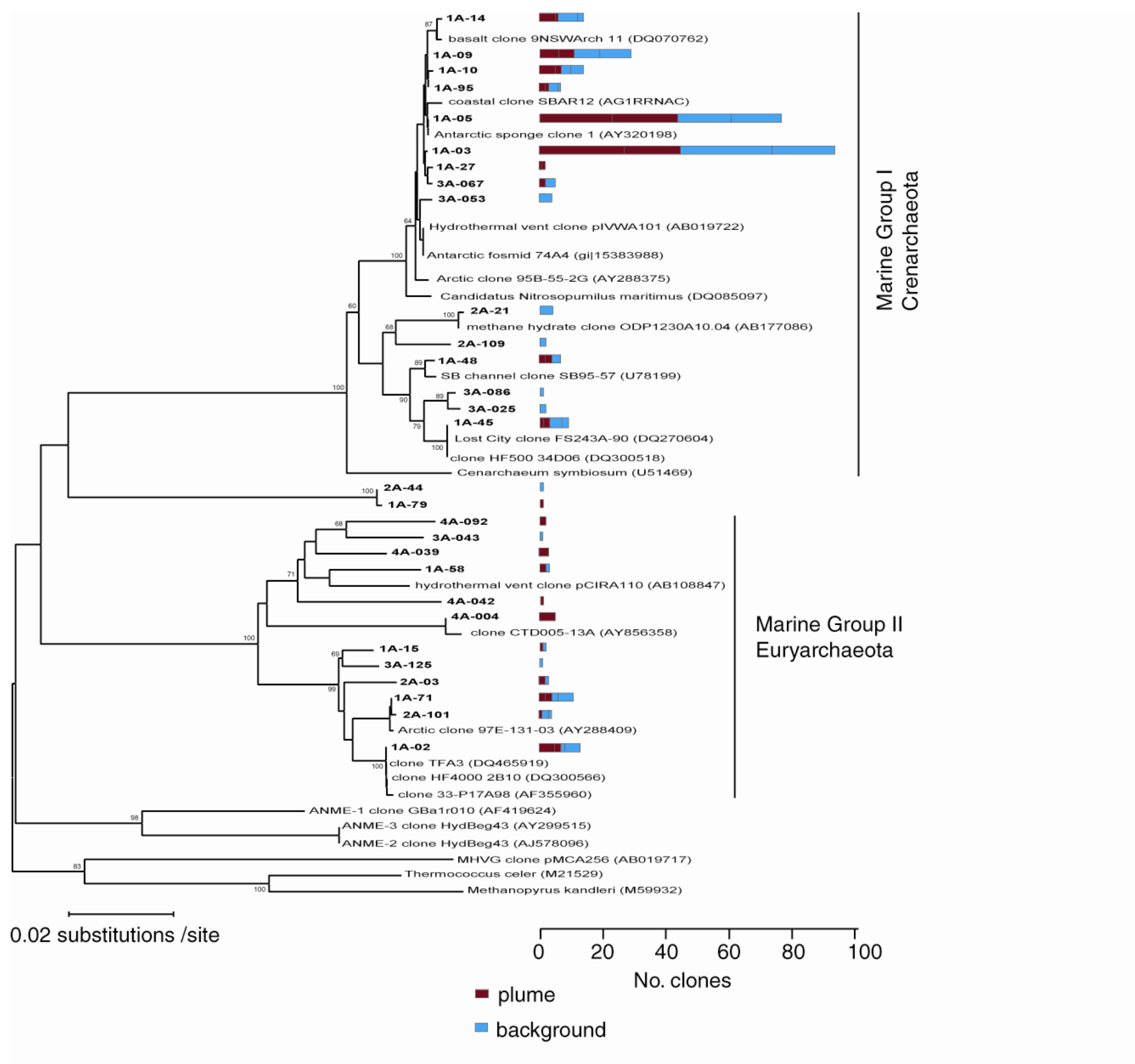
B.

Figure 4.3, Continued (B). Neighbor-joining phylogenetic tree of 16S rRNA genes of α-proteobacteria.



C.

Figure 4.3, Continued (C). Neighbor-joining phylogenetic tree of 16S rRNA genes of β-, δ-, and ε- proteobacteria**.**



D.

Figure 4.3, Continued (D). Neighbor-joining phylogenetic tree of Archaeal 16S rRNA genes.

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V

Direct Identification of a Bacterial Manganese(II) Oxidase: the Multicopper Oxidase MnxG

Abstract

Microorganisms catalyze the formation of naturally occurring Mn oxides, but little is known about the biochemical mechanisms of this important biogeochemical process. We used tandem mass spectrometry (MS/MS) to directly analyze the Mn(II)-oxidizing enzyme from marine *Bacillus* spores, identified as a Mn oxide band with an in-gel activity assay. Nine distinct peptides recovered from the Mn oxide band of two *Bacillus* species were unique to the multicopper oxidase MnxG, and one peptide was from the small hydrophobic protein MnxF. No other proteins were detected in the Mn oxide band, indicating that MnxG (or a MnxF/G complex) directly catalyzes biogenic Mn oxide formation. The Mn(II) oxidase was partially purified and found to be resistant to many proteases and active even at high concentrations of SDS. Comparative analysis of the genes involved in Mn(II) oxidation from three diverse *Bacillus* species revealed a complement of conserved Cu-binding regions not present in well-characterized multicopper oxidases. Our results provide the first identification of a bacterial enzyme that catalyzes Mn(II) oxidation, and suggest that MnxG catalyzes two sequential one-electron oxidations from Mn(II) to Mn(III) and Mn(III) to Mn(IV), a novel type of reaction for a multicopper oxidase.

Introduction

Mn oxides (MnO_2) are reactive minerals that play an important role in the global cycling of many major (C, S) and trace elements (Fe, Co, Pb, Cu, Cd, Cr) in nature (Tebo et al., 2004). Microorganisms are thought to be responsible for the formation of Mn oxides in the environment, catalyzing Mn(II) oxidation rates that are orders of magnitude faster than abiotic Mn(II) oxidation rates (Nealson et al., 1988; Tebo et al., 2004). This microbially-mediated Mn(II) oxidation occurs by an enzymatic pathway (Francis et al., 2001; Rosson and Nealson, 1982) whereby biogenic Mn oxides are precipitated on cell surfaces. Although the importance of microbes in driving the oxidative segment of the Mn cycle has been demonstrated in many environments (Cowen et al., 1986; Cowen et al., 1990; Cowen and Silver, 1984; Fuller and Harvey, 2000; Harvey and Fuller, 1998; Mandernack and Tebo, 1993; Moffett and Ho, 1996; Nealson et al., 1988; Tebo, 1991; Tebo et al., 2004; Tebo et al., 1984; Wehrli et al., 1995), little is known about the biochemical mechanism.

Genetic approaches have elucidated multicopper oxidase (MCO) genes that are required for Mn(II) oxidation in the phylogenetically diverse Mn(II)-oxidizing bacteria *Pseudomonas putida* strain GB-1 (Brouwers et al., 1999), *Leptothrix discophora* strain SS-1 (Corstjens et al., 1997), and *Bacillus sp.* strain SG-1 (van Waasbergen et al., 1996), suggesting a universal mechanism of bacterial Mn(II) oxidation. MCOs are a family of enzymes that use multiple spectroscopically distinct Cu centers as cofactors in coupling the oxidation of substrate to the reduction of O_2 to H_2O (Solomon et al., 1996). MCO substrates include a variety of organic compounds as well as metal ions such as Fe^{2+}, and it has been hypothesized that bacterial MCOs are in fact the direct catalysts of Mn(II)

oxidation. However, a direct link between MCO genes and Mn(II)-oxidizing enzymes has not been made. Repeated efforts to purify native Mn(II) oxidases have failed ((Adams and Ghiorse, 1987a; Okazaki et al., 1997), H.A. Johnson and B.M.T. submitted, D.B. Edwards and B.M.T. unpublished results), as have efforts to produce active Mn(II) oxidase by expressing MCO genes in heterologous hosts (Brouwers et al., 2000b; Francis et al., 2002). Thus biochemical analysis of the Mn(II) oxidase has not been possible, and uncovering the functional role of MCOs in Mn(II) oxidation has been enigmatic. MCOs are involved in a variety of cellular functions (Claus, 2003; Solomon et al., 1996), including iron and copper homeostasis (Huston et al., 2002; Rensing and Grass, 2003; Taylor et al., 2005), siderophore oxidation (Grass et al., 2004), pigment formation (Hullo et al., 2001), and biopolymerization (Sterjiades et al., 1992). The loss of such functions could indirectly lead to the non-Mn(II)-oxidizing phenotype that is observed with MCO mutants, therefore that MCOs directly catalyze Mn(II) oxidation has remained an unproven hypothesis.

In this study, we investigated the role of a MCO in Mn(II) oxidation by marine *Bacillus* spores. Phylogenetically diverse Mn(II)-oxidizing *Bacillus* species have been isolated from a number of environments where Mn(II) oxidation is prevalent, such as coastal sediments (Francis and Tebo, 2002), deep-sea hydrothermal vents (Dick et al., 2006), and the suboxic zone of the Black Sea (G.J.D. and B.M.T., unpublished results). *Bacillus* species oxidize Mn(II) as metabolically dormant spores (not as growing/vegetative cells) via a Mn(II)-oxidizing enzyme (Rosson and Nealson, 1982) located in the exosporium (Francis et al., 2002), an outermost layer of the spore coat. In the model organism *Bacillus* sp. strain SG-1, a cluster of seven genes —designated the

mnx genes—are required for Mn oxidation but show very limited similarity to any database sequences (van Waasbergen et al., 1996). One of these genes, *mnxG*, encodes a predicted protein that has copper-binding amino acid sequences that are signatures of MCOs. Mn(II) oxidation by strain SG-1 proceeds through a Mn(III) intermediate, and *mnxG* is required for both the oxidation of Mn(II) to Mn(III) and Mn(III) to Mn(IV) (Webb et al., 2005a). The possibility that MnxG is the direct catalyst raises interesting mechanistic questions because all well-characterized MCOs catalyze one-electron transfer reactions, whereas the oxidation of Mn(II) to Mn(IV) requires the transfer of two electrons**.** However, as with all other Mn(II)-oxidizing bacteria, the exact role of the MCO in the Mn(II) oxidation reaction remains unknown. Recently, the extensive diversity of marine Mn(II)-oxidizing *Bacillus* spores has been recognized, revealing three major clusters of Mn(II)-oxidizing *Bacillus* species (Dick et al., 2006; Francis and Tebo, 2002). *mnxG* is present in all Mn(II)-oxidizing *Bacillus* species but not in any non-Mn(II)-oxidizers (Francis and Tebo, 2002), therefore *mnxG* is a good genetic marker of Mn(II) oxidation within the genus *Bacillus*. The Mn(II) oxidase can be visualized in these diverse Mn(II)-oxidizing *Bacillus* spores by an SDS-PAGE in-gel activity assay, and its varies dramatically among species (Francis and Tebo, 2002). The Mn(II) oxidase of strain SG-1 hardly enters the resolving gel and is thought to occur as a high-molecular-weight complex, whereas other strains have smaller Mn(II) oxidases of sizes (~100 kDa) that are consistent with being encoded by a single gene (Francis and Tebo, 2002). Here we take advantage of two such strains, PL-12 and MB-7, whose smaller Mn(II) oxidases may be more amenable to genetic and biochemical manipulation. We describe cloning and DNA sequence analysis of the mnx gene cluster from PL-12 and MB-7 and

comparative sequence analysis with SG-1. DNA sequence facilitated tandem mass spectrometric (MS/MS) analysis of the Mn(II) oxidase and led to the most prominent finding of our research – the first identification of a bacterial Mn(II) oxidase. Our results demonstrate that MnxG is a novel MCO that directly catalyzes the oxidation of Mn(II) to Mn(IV) oxide.

Materials and Methods

All molecular cloning was done as described in (Maniatis et al., 1989) unless noted otherwise below.

Growth, DNA extraction, Cloning, and Sequencing. *Bacillus* cultures were grown in K medium (Tebo et al., in press) with shaking (150 rpm) at room temperature. DNA was isolated by phenol/chloroform extraction (Harwood and Archibald, 1990). The PL-12 mnx region was cloned by a combination genomic DNA library screening and inverse PCR, and the MB-7 mnx region was cloned entirely by inverse PCR. The PL-12 library was constructed by digestion of genomic DNA with HindIII, fractionating the HindIII fragments on a 10– 40% (w/v) sucrose-density gradient (Maniatis et al., 1989), selection of 6–8 kb fragments by agarose gel-electrophoresis extraction (Qiagen), ligation with pZERO-2 (Invitrogen), and transformation into E. coli TOP10 competent cells (Invitrogen). Colonies were screened using a ~900-bp digoxygenin-labeled mnxG PCR product (amplified with primers MnxGIf and MnxGIr (Francis and Tebo, 2002)) as a probe with the DIG DNA-labeling and detection kit (Roche). Hybridizations were done at 42°C, washes at 68°C. A 7-kb fragment including the PL-12 mnxG gene and ~3.3 kb downstream was cloned by this method. Inverse PCR products (typically ~1.5 kb) were

generated as described (Ochman et al., 1988) using the restriction enzyme HaeII and polymerase Pfu Turbo (Stratagene), and cloned using the TOPO TA cloning kit (Invitrogen). The PL-12 and MB-7 mnx regions were cloned by primer walking with successive cycles of inverse PCR, cloning, and DNA sequencing.

Heterologous Expression. *mnxG* was PCR-amplified with Pfu Turbo (Stratagene) and primers PL12mnxGf (ggtctcacatgttacgaaaatttcatg) and PL12mnxGr (ttatgccttttcttcattgtcc). The PCR cycling was 1 cycle at 95°C (2 min), 30 cycles of 95°C (30 s), 47°C (30 s), 72°C (4 min), followed by 1 cycle at 72°C (10 min). The PCR product (~3.7 kb) was TOPO cloned into pCR2.1 (Invitrogen), excised with BsaI and EcoRI, subcloned into pET21d (Novagen) at the NcoI/EcoRI site, and transformed into *E. coli* strain BL-21(DE3) (Novagen). Cultures were incubated for 8–9 hours at 37°C (OD600 = 0.89) and *mnxG* expression was induced with IPTG for either 2 or 4 hours. The induced *E. coli* was french pressed (20,000 psi) and assayed for Mn(II)-oxidizing activity as described below.

Preparation of spores and purification of the Mn(II) oxidase. Spores were purified and exosporium isolated as described (Dick et al., 2006). Solubilization was tested with various concentrations of deoxycholate, TritonX-100, CHAPS, Tween-20, NP-40, and SDS (0.1% - 5%), NaCl (0 – 0.5M), and glycerol (0 or 10%). For protein purification, crude exosporium extracts were digested with sequencing-grade trypsin (Promega) in 50 mM Hepes, pH 7.8 for one hour at 37°C, then solubilized in 2% SDS, 10% glycerol, 1 mM DTT, and 50 mM HEPES, pH 7.8 for two hours. Insoluble complexes were removed by ultracentrifugation at 86,000g. Solubilized samples were then fractionated at room temperature on a superpose 6 HR 10/30 size-exclusion column

(Amersham Pharmacia) at a flow rate of 0.3 ml/minute using an Akta FPLC (Amersham Pharmacia). 1-ml fractions were collected and assayed for Mn(II)-oxidizing activity, and active fractions were concentrated by ultrafiltration (50-kDa MWCO, Millipore).

Mn(II) oxidation assays and specific activity determination. Mn(II) oxidation was assayed in reactions containing 800 μM $MnCl_2$ and 20 mM Hepes pH 7.8. The quantity of MnO_2 formed was determined by the leukoberbelin blue (LBB) method (Krumbein and Altmann, 1973; Tebo et al., in press) using potassium permanganate as a standard. For rate determinations for specific activity, time points were analyzed every hour for four hours. Protein concentrations were determined with the DC protein assay (Bio-Rad). The protein concentration of the partially purified fraction was below the detection limits of our assay/standard curve, so we present specific activity per absorption at 750 nm (after protein assay and color development) rather than per mg protein.

SDS-PAGE analysis and in-gel digestion. SDS-PAGE analysis and the in-gel Mn(II) oxidation assay were done as described (Francis and Tebo, 2002). For strain SG-1, a 2.5% stacking gel was required to allow the Mn(II) oxidase to enter the gel. The darkest portion of the Mn(II) oxidase bands were excised from the gel with sterile scalpel, washed with 200 μL of distilled H_2O, and vortexed for 10 minutes before removal of water. Destaining solutions A and B (SilverQuest Silver Staining Kit, Invitrogen) were mixed in equal proportions and used to destain the Mn oxide bands (100 μL/band). The sample was then vortexed at room temperature for 15 minutes, the supernatant removed, and washed again with water. Samples were then taken through two cycles of mixing with 200 μL of 25 mM ammonium bicarbonate / 5 mM dithiothreitol (DTT) / 50% acetonitrile (ACN), vortexing for 10 minutes and removing

supernatant. Finally, the gel piece was dehydrated with 100 µL of ACN (room temperature, 10 minutes), the ACN was removed, and 400 ng ice-cold trypsin (Promega) in 25 mM ammonium bicarbonate / 5 mM DTT solution was added to the sample and set on ice for 30 minutes. After complete rehydration, the excess trypsin solution was removed, replaced with fresh 25 mM ammonium bicarbonate / 5 mM DTT and left overnight at 37°C. The peptides were extracted by addition of 2 µL of 2% trifluoroacetic acid (TFA) and vortexing at room temperature for 30 minutes. The supernatant was extracted and saved. 20 µL of 20% ACN / 0.1% TFA was added to the sample, which was vortexed again at room temperature for 30 minutes. The supernatant was extracted and combined with the supernatant from the first extraction.

LC-MS/MS analysis. All electrospray ionization experiments were performed by Justin Torpey at the UCSD Biomolecular Mass Spectrometry Facility using a QSTAR-XL hybrid mass spectrometer (AB/MDS Sciex) interfaced to a nano-scale reverse-phase HPLC (Famos/Ultimate/Switchos; LC Packings) using a 10 cm x 75 µm 218MS column (GraceVydac) packed with 3 µm C18 beads. The buffer compositions were as follows: buffer A: 98% H_2O, 2% ACN, 0.1% formic acid, 0.01% TFA; buffer B: 98% ACN, 2% H_2O, 0.1% formic acid, 0.01% TFA. 10 µL was injected by the Famos onto the Switchos C18 pre-column (5 cm x 300 µm, LC Packings) using buffer A at a flow rate of 30 µL/min. After washing for 3 minutes, the Switchos valve was switched and the peptides backflushed onto the analytical column and eluted with a 20-minute linear gradient from 10 to 40% buffer B at a flow rate of ~200 nL/min. Peptides from keratin contamination and trypsin autolysis were identified and excluded from further

analyses. Some samples were re-analyzed with exclusion lists of previously observed keratin and/or trypsin autolysis peaks.

Database Search and Analysis. The translated mnx gene sequences were used to search the MS/MS data for the presence of mnx peptides using Analyst QS 1.1/ProID 1.1 (Tang et al., 2005) and BioAnalyst 1.1.5/ProBlast 1.1 (Schevenko et al., 2001) (Applied Biosystems). To test for statistically meaningful protein identifications using ProID and ProBLAST, the MS/MS spectra were also tested against a database in which the mnx gene sequences were inserted into SwissProt database. Each mnx-related MS/MS spectrum was then manually inspected to verify the accuracy of the search results. To identify peptides not present in any databases, all 74 MS/MS spectra obtained from strain PL-12 were also manually *de novo* sequenced.

Results

Comparative analysis of the ultrastructure, mnx region, and Mn(II) oxidase from diverse *Bacillus* species. The size of the Mn(II) oxidase varies dramatically among representatives of the three major phylogenetic clusters of Mn(II)-oxidizing *Bacillus* species, strains PL-12, MB-7, and SG-1 (Fig. 5.1) (Francis and Tebo, 2002). TEM shows prominent differences in the extent of exosporium between spores of these three strains (Fig. 5.2). To investigate the genetic basis for variation of Mn(II) oxidase size, we cloned and sequenced the mnx region from *Bacillus* spp. strains PL-12 and MB-7, and compared them with SG-1. The organization of the mnx operon varies between the three strains: *mnxC* is missing from the mnx region of both PL-12 and MB-7, and

mnxA and *mnxB* are inverted in PL-12 (Fig. 5.3A). *mnxG* is the most highly conserved gene (58% predicted amino acid identity between the three strains), whereas other genes show 20–47% amino acid identity (Table 5.1). The length of the *mnxG* gene is nearly identical in all three strains, therefore the difference in the size of the Mn(II) oxidases (Fig. 5.1) cannot be explained by differing sizes of the genes that are suspected to encode them.

The predicted amino acid sequence of MnxG contains the four consensus Cu-binding regions found in all MCOs (Solomon et al., 1996; van Waasbergen et al., 1996) (labeled #2-5 in Fig. 5.3), and a putative fifth consensus Cu-binding region near the C-terminus of the protein (Fig. 5.3 region 6) (Francis and Tebo, 1999; van Waasbergen et al., 1996). Our comparative analysis reveals that this fifth Cu-binding region is conserved among the three *Bacillus* spp., consistent with a critical functional role for those amino acids. Another prominent region of conservation occurs at the C-terminus of MnxF (Fig. 5.3A region 1). Although MnxF contains only 30% amino acid identity between the three stains, 11 of 13 amino acids are conserved in one stretch at the C-terminus. Surprisingly, this stretch includes sequence that resembles a MCO Cu-binding region (Fig. 5.3B region 1). MnxG also has at least seven homologues of MCO Cu-coordinating amino acids in regions that are less similar to the consensus MCO Cu-binding motifs (Fig. 5.3B A-D). Based on homology of MCO Cu-binding regions, the extra Cu-binding amino acids found in MnxF and MnxG are predicted to be ligands for all three spectroscopic types of Cu atoms present in multicopper oxidases (Fig. 5.3B).

Sequence analysis identified several interesting genes downstream of *mnxG* in PL-12 (data not shown). Following *mnxG* is an open reading frame (ORF) coding for a

predicted 25-kDa protein with limited sequence similarity (~30% amino acid) to MnxD. The position of this gene and the similarity to *mnxD* suggest that it is part of the mnx operon and should be designated *mnxH*. One striking feature of the predicted MnxD/H proteins is their high lysine content (25% and 30% respectively); in one stretch of MnxH sequence, 15 of 30 predicted amino acids are lysine**.** Sequencing the fragment downstream of *mnxG* on a previously cloned fragment from SG-1 (van Waasbergen et al., 1996) revealed that *mnxH* is not present in that strain at that chromosomal location. Further downstream of the mnx region in PL-12 are two hypothetical proteins, followed by an aluminum-resistance protein, ExsB, and an ORF with similarity to a pyroquinoline-quinone (PQQ) synthesis gene.

Partial purification of the Mn(II) oxidase and Heterologous Expression of *mnxG* from *Bacillus* sp. strain PL-12. We attempted to purify the Mn(II) oxidase from strain PL-12. The Mn(II) oxidase is insoluble, so we tested a variety of solubilizing detergents (deoxycholate, TritonX-100, CHAPS, Tween-20, NP-40, SDS, etc.) at various concentrations and conditions (NaOH, NaCl, glycerol, DTT, EDTA). Only SDS solubilized Mn(II)-oxidizing activity, with high concentrations (up to 2%) being most effective. The Mn(II) oxidase remained active in the presence of SDS, a characteristic observed with some other MCOs (Diamantidis et al., 2000) and Mn(II) oxidases (Francis et al., 2001; Okazaki et al., 1997). Efforts to remove the SDS or exchange it for another detergent and maintain activity were unsuccessful, thereby limiting purification options.

Another interesting property of the Mn(II)-oxidizing activity from PL-12 is that it is resistant to many proteases in solution. Of the proteases tested (pronase, trypsin, Glu-C, Arg-C, Asp-N, Lys-C), only pronase was able to disrupt Mn(II)-oxidizing activity as

detected by the in-gel assay. We used this trypsin resistance as a purification step; after digestion of crude exosporium extract with trypsin, Mn(II)-oxidizing activity was unaffected or in some cases even enhanced, whereas nearly all other exosporium proteins were digested (Fig. 5.4). Following solubilization and size-exclusion chromatography of the trypsin-digested exosporium, specific activity increased >50-fold from the solubilized crude exosporium (2 nmol Mn hr^{-1} $A750^{-1}$) to the most pure fraction (119 nmol Mn hr^{-1} $A750^{-1}$) (we report specific activity per A750 rather than per mg protein because the protein concentration in the partially-purified fraction was below our detection limits, see *Materials and Methods*). In this partially purified fraction, the coomassie band corresponding to the Mn(II) oxidase is faint, and multiple other proteins remain.

Heterologous expression of the PL-12 *mnxG* (suspected to encode the Mn(II) oxidase) in *E. coli* produced a protein of the predicted size (137 kDa), however no Mn(II)-oxidizing activity was detected in crude cell extracts of the recombinant *E. coli* (data not shown).

MS/MS identification of the Mn(II) oxidase. We analyzed the SDS-PAGE Mn oxide bands of *Bacillus* spp. strains PL-12, MB-7, and SG-1 by in-gel tryptic digestion, followed by nano-scale liquid chromatography mass spectrometric (nanoLCMS) with tandem mass analysis (MS/MS). Although no pertinent peptides were recovered from the crude PL-12 exosporium, five distinct peptides unique to MnxG were detected from the partially purified PL-12 Mn(II) oxidase (Fig. 5.5, Table 5.2). Four different MnxG peptides and one MnxF peptide (the C-terminal tryptic peptide) were identified in the Mn oxide band of strain MB-7 (Table 5.2). The MnxG peptides detected from PL-12 and MB-7 cluster towards the C-terminus of the predicted protein sequence (Fig. 5.6). No

other *Bacillus* protein peptides were detected in the Mn oxide band of PL-12 or MB-7. A higher molecular weight band (~ 140-kDa) often present in the in-gel Mn(II) oxidation assay of strain MB-7 (Fig. 5.1) and occasionally PL-12 (data not shown) did not yield any detectable peptides. No peptides were recovered from the SG-1 Mn oxide band.

The overall coverage of the MnxG protein sequence with peptides identified by MS/MS was low (Fig. 5.6), and there was a high incidence of peptides from trypsin autolysis (trypsin digesting itself) and semi-tryptic cleavages (having a tryptic cleavage at one end and a non-specific cleavage at the other). 74 MS/MS spectra obtained from strain PL-12 were de novo sequenced, and 35 spectra were of sufficient data quality for interpretation. 30 of these peptides were from trypsin, and only five spectra were from MnxG. Two of five MnxG peptides were semi-tryptic: L.EFVLLLHDGVR produced by cleavage after leucine and arginine, and K.APRPPLGIV produced by cleavage after lysine and valine. One of the MB-7 peptides we recovered was also semi-tryptic (Table 5.2).

Discussion

Multicopper oxidases (MCOs) are a large family of enzymes with over 500 putative homologs in all three domains of life (Stoj and Kosman, 2006; Taylor et al., 2005). Though all MCOs couple the oxidation of substrate to the reduction of O_2 to H_2O, their substrates (organics vs. metals) and specificity (narrow vs. broad) vary widely, and their cellular roles are functionally diverse, ranging from trace metal uptake and homeostasis to lignin degradation and antibiotic biosynthesis. MCOs have long been

known to be involved in bacterial Mn(II) oxidation, a process of broad environmental importance, but unraveling their role in bacterial Mn(II) oxidation has proven enigmatic. Here we present the first evidence that a bacterial MCO directly catalyzes Mn(II) oxidation: MS/MS analysis of SDS-PAGE purified Mn(II) oxidase from spores of two *Bacillus* spp. identified nine different peptides unique to the MCO MnxG and one peptide unique to the small hydrophobic protein MnxF. No other *Bacillus* peptides were found in the Mn oxide bands from these two strains, indicating that MnxG is the dominant protein in the gel at the site of Mn oxide biomineralization and therefore is the direct catalyst of Mn(II) oxidation**.** The detection of one MnxF peptide in the MB-7 Mn oxide band from strain MB-7 suggests that this small hydrophobic protein is associated with the Mn(II) oxidase MnxG. While the nature of this protein-protein interaction is unknown, the presence of a putative copper-binding motif in MnxF suggests that it may be involved either directly in the Mn oxidation reaction, or in the delivery of Cu to the MCO as in other Cu proteins (O'Halloran and Cizewski Culotta, 2000). Alternatively, the hydrophobic nature of MnxF may reflect a structural role in the spore coat, perhaps in anchoring the Mn(II) oxidase to the exosporium.

MS/MS peptide coverage of the MnxG sequence was low and clustered to the C-terminus of the protein (Fig. 5.6), perhaps suggesting that the N-terminus is either not translated (i.e. an internal start codon is used), post-translationally modified, or is resistant to trypsin digestion. The lack of coverage of MnxG can likely be explained at least in part by the low abundance of the Mn(II) oxidase; there is no coomassie-stainable band corresponding to the Mn oxide band in crude cell extracts, and it is only barely visible in the partially purified fraction. The high incidence of non-specific cleavages

and trypsin autolysis peaks in the MS/MS spectra are also consistent with a low concentration of MnxG protein. These observations, including the absence of a clear coomassie stainable band, suggest that there was at most 10-20 ng (~100-200 fmol) of MnxG protein present in the gel band. Another possible explanation for the low MS/MS coverage is that MnxG is resistant to tryptic digestion. Trypsin specifically cleaves proteins at arginine and lysine; the relatively high occurrence of non-specific cleavages that we observed suggests that peptides were produced with low efficiency. Although there are many tryptic cleavage sites (arginine or lysine) in MnxG, tryptic digestion of crude exosporium in solution does not inhibit Mn(II)-oxidizing activity. The clustering of peptides detected by MS/MS at the C-terminus may indicate that these sites are more accessible whereas others are buried within the protein. Protease resistance has been observed in other exosporium proteins (Keim et al., 2006).

No peptides were detected from the Mn oxide band of strain SG-1. This is most likely due to extremely low levels of the Mn(II) oxidase in the gel as a result of its enormous apparent molecular weight, which limits its entry into the SDS-PAGE resolving gel**.** The larger size of the SG-1 Mn(II) oxidase cannot be accounted for by the size of the predicted protein product of *mnxG*, which is nearly identical to that of strains PL-12 and MB-7 (138 kDa). The SG-1 Mn(II) oxidase may occur as a high-molecular-weight complex because of the association of additional subunits and/or proteins, or because of differences in the overall structure of the exosporium. TEM shows that these three stains have prominent differences in the extent of exosporium that is present: PL-12 has a large exosporium SG-1's is less prominent, and MB-7's is not detectable (Fig. 5.2). Differences in exosporium structure such as extent of protein-protein cross-linking

or glycosylation could explain the varied migration of the Mn(II) oxidase through SDS-PAGE. Little is known about the composition, structure, or function of the exosporium in diverse *Bacillus* species, yet as the interface between spore and environment it undoubtedly plays important roles in the ecology and biogeochemical impact of spores.

Taken together with our previous findings that MnxG is required to oxidize Mn(II) to Mn(III) and Mn(III) to Mn(IV) (Webb et al., 2005a), our results indicate that MnxG directly catalyzes Mn(II) to Mn(IV) via a Mn(III) intermediate. MnxG is the first MCO demonstrated to directly catalyze two sequential one-electron oxidations of one substrate molecule. Well-characterized MCOs such as Fet3p, ascorbate oxidase, laccase, and human ceruloplasmin (hCp) oxidize each substrate by a single electron that is accepted by the enzyme at the type-1 Cu center. Electrons are then passed to the trinuclear cluster, consisting of one type-2 and two type-3 Cu sites, where four electrons (from four substrate molecules) reduce O_2 to $2H_2O$ (Solomon et al., 1996). The two-electron oxidation reaction catalyzed by MnxG raises interesting mechanistic questions: How does MnxG catalyze two energetically distinct oxidation reactions with products and reactants that demand different ligand chemistry? Does each oxidation step occur at the same site, or is Mn(III) transferred to a different active site prior to oxidation to Mn(IV)? Does MnxG contain extra redox active Cu cofactors to handle two energetically distinct oxidations?

The ferroxidases Fet3p and hCp may provide some insights into the mechanism of Mn oxidation. Fe(II) is oxidized to Fe(III), and the redox potential of the oxidation site is modulated via the relative affinity of the protein for Fe(II) and Fe(III) (Quintanar et al., 2004). After oxidation, Fe(III) is translocated to a “holding site” where it is then donated

to transferrin (hCp) or Ftr1p (Fet3p) (Bonaccorsi di Patti et al., 1999; Kwok et al., 2006; Murphy et al., 1997; Wang et al., 2003). Such a translocation may occur in the Mn(II) oxidase if the Mn(II) and Mn(III) oxidation sites are distinct, but how that might relate to electron transfer is unclear. Ferroxidases exhibit high specificity for Fe that has been attributed to a substrate binding pocket (Taylor et al., 2005), and of those that have been tested to date (*P. aeruginosa* MCO, CueO, and Fet3p) none are able to oxidize Mn(II) (Huston et al., 2002; Kim et al., 2001). In contrast, laccases lack a substrate binding pocket, exhibiting broad substrate specificity that correlates with the substrate's oxidation potential (Solomon et al., 1996). Fungal laccases are capable of oxidizing Mn(II) to Mn(III) (Schlosser and Höfer, 2002). In some cases Mn(IV) oxides are produced (Miyata et al., 2006), but it is unclear whether the Mn(III) to Mn(IV) step is enzymatic. The only other MCO thought to directly catalyze multiple-electron oxidations of substrate is phenoxazinone synthase, which catalyzes the biosynthesis of Actinomycin D via a series of three two-electron oxidations (Barry et al., 1989). However, the enzymatic mechanism of this multi-electron transfer is not understood.

Although MnxG directly oxidizes Mn(II) and is also required for Mn(III) oxidation, it is possible that MnxF—which was also detected in one of the Mn oxide bands—participates in the oxidation of Mn(II) and/or Mn(III). There could also be unknown cofactors that are involved in the Mn oxidation reaction. We found a pyrroloquinoline quinone (PQQ) synthesis gene in close proximity to the mnx region in strain PL-12. PQQ is a quinone that is involved in electron transfer reactions (Anthony, 1996; Duine, 1999) and was recently shown to be involved in Mn(II) oxidation by Mn(II)-oxidizing α- and γ-proteobacteria (H.A. Johnson and B.M. Tebo, submitted).

Another gene of interest that was found immediately downstream of *mnxG* in strain PL-12 shows predicted amino acid sequence similarity to MnxD. Its position immediately downstream of *mnxG* and its similarity to MnxD suggest that this gene is likely a part of the Mn(II) oxidation operon, and we have designated it *mnxH*. A striking feature of both MnxD and MnxH is their high lysine content (25 and 30%, respectively). Lysine-rich proteins play an important role in biomineralization of silica in diatoms (Kroger et al., 1999; Poulsen et al., 2003), and it is conceivable that MnxD and MnxH could play a similar role in the formation of the initial Mn oxide nanoparticles if the positively charged lysine residues interact with negative charges on the MnO_2 surface.

The MnxF/G proteins contain a unique set of Cu-binding regions not present in any characterized MCOs but most similar to hCp (Fig. 5.3). Cu-binding region 6 (Fig. 5.3) is also of interest because transposon insertions in the extreme 3' end of *mnxG* still abolish Mn(II)-oxidizing activity (van Waasbergen et al., 1996), suggesting that this Cu-binding region is crucial. Conserved histidines within these regions are required for activity in other MCOs (Taylor et al., 2005), and structural data has confirmed their role in binding Cu (Roberts et al., 2002; Taylor et al., 2005; Zaitseva et al., 1996). Based on sequence homology, the extra Cu-binding ligands of MnxF/G are predicted to coordinate all three different Cu types. Although there are not enough extra ligands for full coordination of functional type-1 Cu site or trinuclear cluster, the conservation of these histidines suggests coordination of redox-active Cu ions. Other Cu ligands that have not yet been detected could lie elsewhere in the MnxG amino acid sequence. Perhaps these putative extra Cu ligands are evidence of extra Cu cofactors that are required for the unique two-electron oxidation catalyzed by MnxG. hCp is the only well-characterized

MCO known to contain more than four Cu cofactors; it has two extra Cu ions that are both type-1. One is not redox-active and therefore catalytically irrelevant (Machonkin and Solomon, 2000), and the function of the other type-1 Cu, though it is redox active, is unclear. Other MCOs with extra Cu ions include CueO (*E. coli*) where the extra Cu ion is involved in regulation (Roberts et al., 2003), and phenoxazinone synthase, where the extra Cu ion is thought to stabilize quaternary structure (Smith et al., 2006). These cases are distinguished from MnxG because the Cu ions are coordinated by histidines and methionines that are not homologous to the consensus MCO Cu-binding regions.

Our results identify an enzyme that drives the oxidative segment of the Mn cycle, a biogeochemical process with important environmental consequences. MnxG's unusual complement of putative Cu ligands and the two-electron reaction that it catalyzes suggest that it is a novel MCO that may provide insights into the mechanism and evolution of this important class of enzymes. Determination of the number of Cu ions bound by MnxG and elucidation of their role in the biochemical mechanism of Mn oxidation awaits purification of active Mn(II) oxidase. The limited abundance and solubility of MnxG make purification of the native enzyme from *Bacillus* spores difficult, and so far efforts to express MnxG in *E. coli* to have failed to yield an active Mn(II) oxidase. The myriad obstacles to heterologous expression are well-documented, however many of these difficulties might be overcome by expression in a more closely related host, such as another *Bacillus* species.

Finally, another major enigma clouding Mn(II) oxidation by marine *Bacillus* spores is the function that this process serves for the spore. Mn is required for sporulation in *Bacillus* species (including those not known to oxidize Mn) (Jakubovics

and Jenkinson, 2001), but a connection between this requirement and Mn oxidation has not been made. Bacterial Mn(II) oxidation has also been suggested as a strategy for accessing refractory organic carbon (Sunda and Kieber, 1994). Other possible functions include storage of an oxidant and protection from predation, ionizing radiation, or reactive oxygen species (ROS) (Tebo et al., 1997b). Mn generally plays an important role in biology in protection against ionizing radiation/ROS (Daly et al., 2004; Inaoka et al., 1999; Jakubovics and Jenkinson, 2001), and MCOs have been implicated in melanization for UV protection in the *Bacillus subtilis* spore coat (Hullo et al., 2001). Whatever the function of Mn(II) oxidation, it is clear that *Bacillus* spores should be considered reactive catalysts of biogeochemical processes rather than merely dormant/inert structures.

Table 5.1. Amino acid sequence similarity of Mnx proteins from *Bacillus* sp. strains SG-1, PL-12, and MB-7.

Predicted protein	% amino acids identical in all three strains
MnxA*	46.6*
MnxB	20.0
MnxC**	**
MnxD	34.3
MnxE	43.2
MnxF	30.3
MnxG	57.8

* present only in strains PL-12 and SG-1
** present only in strain SG-1

Table 5.2. Peptides identified by MS/MS analysis of the Mn(II) oxidase

Strain	Protein	Peptide	Amino acid position
PL-12	MnxG	AITGENVILR	1011-1020
PL-12	MnxG	SFGTFGAFVAESR	986 - 998
PL-12	MnxG	EFVLLLHDGVR	1024 - 1034
PL-12	MnxG	MPHILDGDAFQLVTR*	508 - 522
PL-12	MnxG	APRPPLGIV	402 - 410
MB-7	MnxG	EFVLVMYDGAR	1024 - 1034
MB-7	MnxG	ANQGDVVEINLTSR	884 - 897
MB-7	MnxG	ISLHAQLLDYDVK	927 - 939
MB-7	MnxG	SLGTFGAFIAEPK	986 - 998
MB-7	MnxF	IPELRDDF	95 - 102

*only the five C-terminal residues show unambiguous MS/MS sequence for this peptide

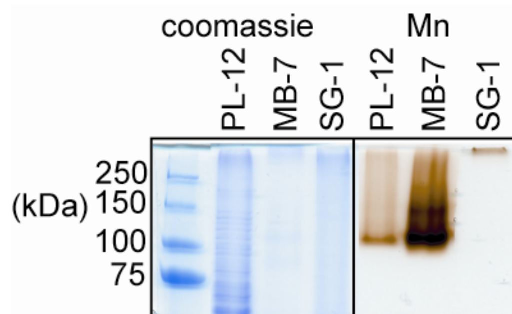


Figure 5.1. SDS-PAGE of exosporium extracts from three *Bacillus* strains, stained with coomassie (left) and an in-gel Mn(II) oxidation activity assay (right).

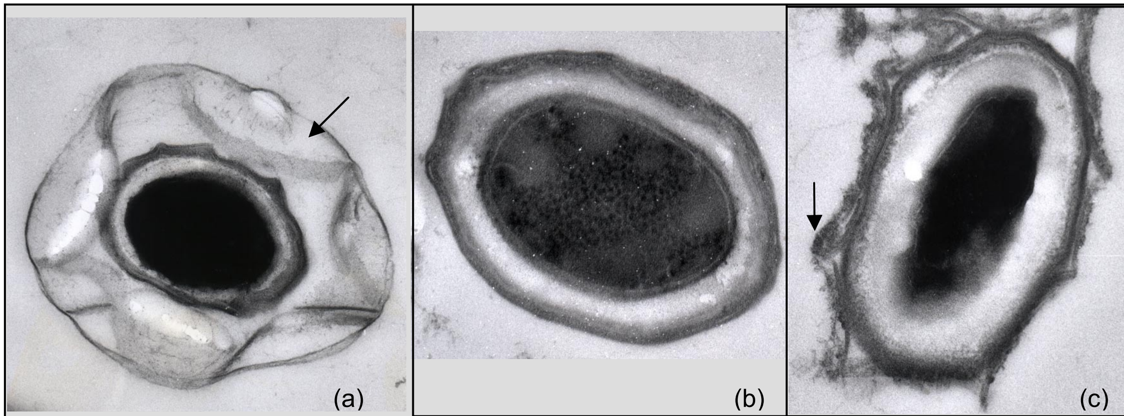
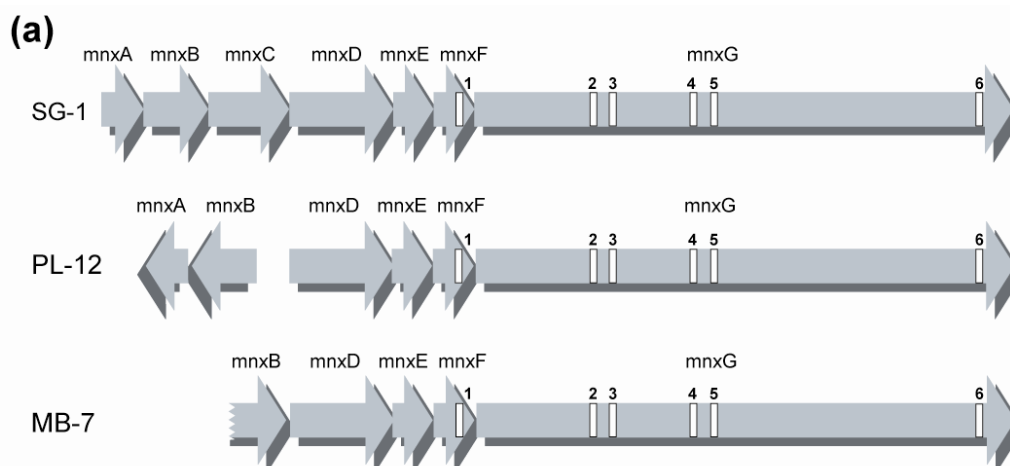


Figure 5.2. TEM of spores from *Bacillus* species strains PL-12 (a), MB-7 (b), and SG-1 (c). Arrows indicate exosporium, which is not detectable for strain MB-7. (Courtesy of Terry Beveridge, University of Gelph).

Figure 5.3. (a) Schematic representation of mnx region genes of *Bacillus* sp. strains SG-1, PL-12 and MB-7. Locations of Cu-binding regions are indicated by open rectangles and numbered according to (b). (b) Alignment of putative mnx Cu-binding region amino acid sequences (1-6) to consensus Cu-binding sequence motifs found in all MCOs (I-IV). The consensus sequences shown are for the well-characterized MCOs laccase (lac), ascorbate oxidase (AO), ceruloplasmin (hCp), and Fet3p, as well as putative MCOs involved in Mn(II) oxidation in *Pseudomonas putida* (CumA) and *Leptothrix discophora* (MofA). AA# indicates the number of the first amino acid of each block. Note that the order of the MnxG Cu-binding regions (2, 3, 4, 5) is different than all other MCOs (III, IV, I, II). Consensus MCO Cu-binding motifs are highlighted in black; mnx regions 1 and 6 are 5th and 6th Cu-binding regions that are not found in other MCOs. Additional Cu-binding regions present in human ceruloplasmin and MnxG (only SG-1 shown here) are shown in A-D and ligands are highlighted with gray. The type of Cu ion coordinated by each ligand (based on characterized MCOs) is indicated with T1, T2, and T3.



(b)

I.

	AA#	Sequence (T1, T2, T3)
Lac	418	H P F H L H G H T F
AO	445	H P W H L H G H D F
hCp	994	H T V H F H G H S F
Fet3p	413	H P F H L H G H A F
CumA	391	H P I H L H G M S F
MofA	1174	H P V H F H L L N V
2. SG-1 MnxG	281	H V F H Y H V H Q W
PL-12 MnxG	277	H V F H Y H V H Q W
MB-7 MnxG	281	H S F H Y H V H Q W
6. SG-1 MnxG	1132	H T F H L H G H Y W
PL-12 MnxG	1131	H T F H L H G H Y W
MB-7 MnxG	1131	H T F H I H G H Y W
B. SG-1 MnxG	718	H S F N V H G L R W
hCp	656	H G I Y F S G N T Y
hCp	295	H A A F F H G Q A L

II.

	AA#	Sequence (T3 T1 T3, T1, T1)
	466	N P G P W F L H C H I D F H L E A - - G F A
	499	N P G V W A F H C H I E P H L H M - - G M G
	1032	T P G I W L L H C H V T D H I H A - - G M E
	476	N P G V W F F H C H I E W H L L Q - - G L G
	435	N P G T W M F H C H V I D H M E T - - G L M
	1272	Y Q W E Y V W H C H I L G H E E N - - D F M
3.	327	A I G D A I I H C H L Y P H F G I - - G M W
	327	A I G D S I I H C H L Y P H F G I - - G M W
	327	T F G D V I L H C H L Y P H F G V - - G M G
A.	1179	F P G D Y M Y R S G N I Q W D I E Q - G M W
C.	966	F P V G A - - - C - - - - - - - - - - - G M W
	691	T E G T F N V E C L T T D H Y T G - - G M K
	330	N P G E W M L S C Q N L N H L K A - - G L Q

III.

	AA#	Sequence (T2, T3)
Lac	83	T S I H W H G F F Q K G T N W A D G
AO	57	V V I H W H G I L Q R G T P W A D G
hCp	117	Y T F H S H G I T Y Y K E H E G A I
Fet3p	78	T S M H F H G L F Q N G T A S M D G
CumA	93	T T I H W H G I R L P L E M - - D G
MofA	302	S N I H L H G G D T P W I S - - D G
4. SG-1 MnxG	526	A G M H I H F V K F D V L V N - D G
PL-12 MnxG	526	N G F H I H F V K F D V L A C - D G
MB-7 MnxG	527	A S F H V H F V K F D V L V S - D G
1. SG-1 MnxF	74	W H I H I H N I E V F F Y I E K S T
PL-12 MnxF	77	W Y I H I H N I E V F F Y I E R P G
MB-7 MnxF	75	W Y I H I H Q I E V F F Y I Q Q K G
D. SG-1 MnxG	78	T S M H F Q E A E Y D V L T S - D G
SG-1 MnxG	925	I S L H P Q L I Q Y D V K T S - S G

IV.

	AA#	Sequence (T3, T3)
	129	W Y H S H L S T Q Y C D G L
	102	F Y H G H L G M Q R S A G L
	178	I Y H S H I D A P K D I A S
	124	W Y H S H T D G Q Y E D G M
	136	W Y H P H V S S S E E L G R
	384	W Y H D H T I G V T R L N V
5.	573	F F H D H L F A V Q H Q Q H
	573	F F H D H L F A N S H Q Q H
	574	F F H D H L F A N F H Q Q H

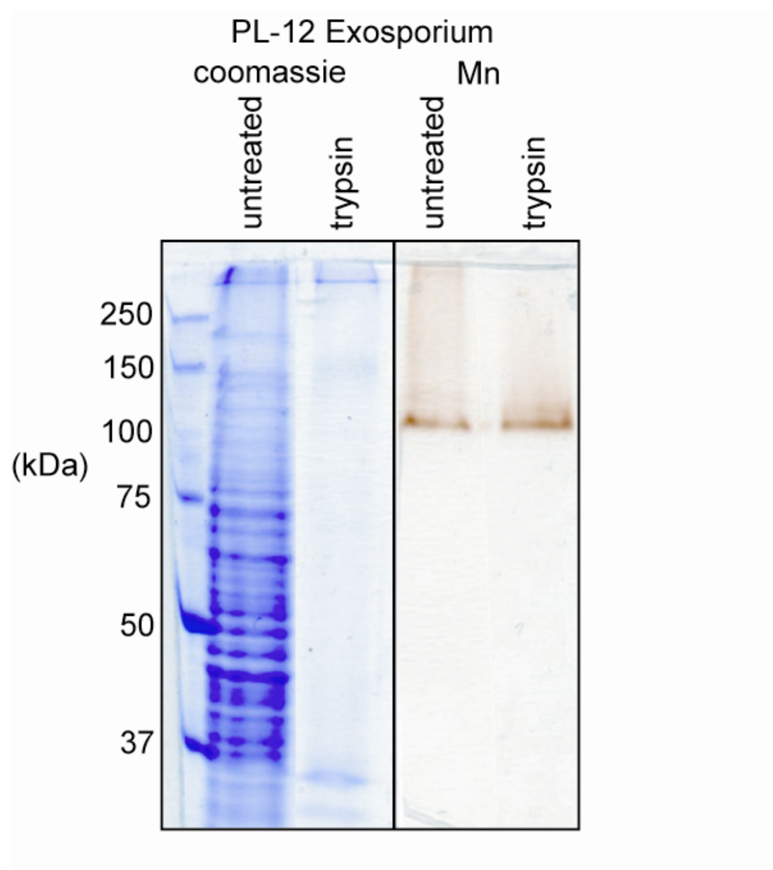


Figure 5.4. SDS-PAGE of PL-12 exosporium stained with coomassie (left) and Mn (right). Digestion with trypsin degrades most exosporium proteins, but Mn(II)-oxidizing activity is not inhibited.

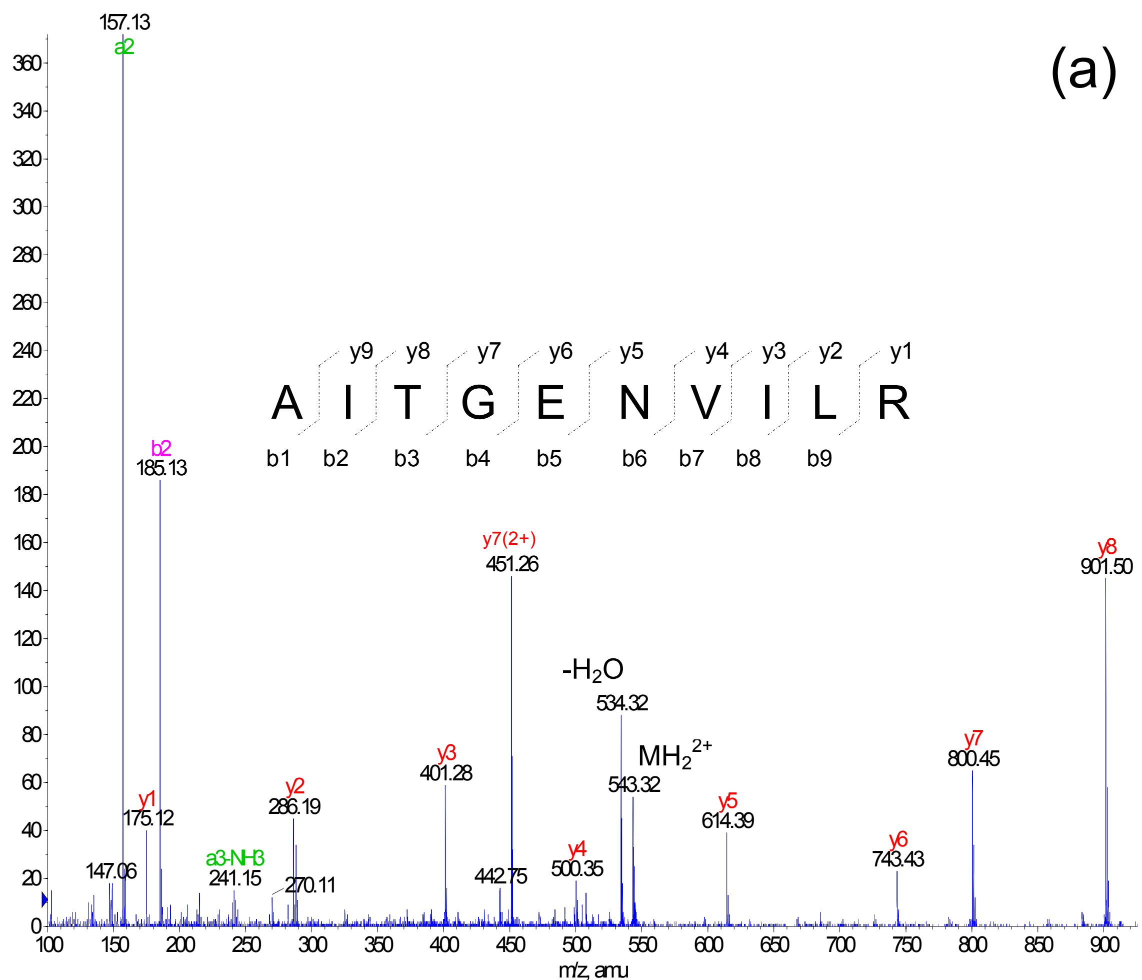


Figure 5.5. Representative MS/MS of peptides obtained from in-gel tryptic digest of SDS-PAGE purified Mn oxidase from strain PL-12. Fragment ion types are indicated for amide backbone cleavages of (A) *m/z* 543.3, (B) *m/z* 649.4, and (C) *m/z* 688.3 as described previously (Roepstorff and Fohlman, 1984).

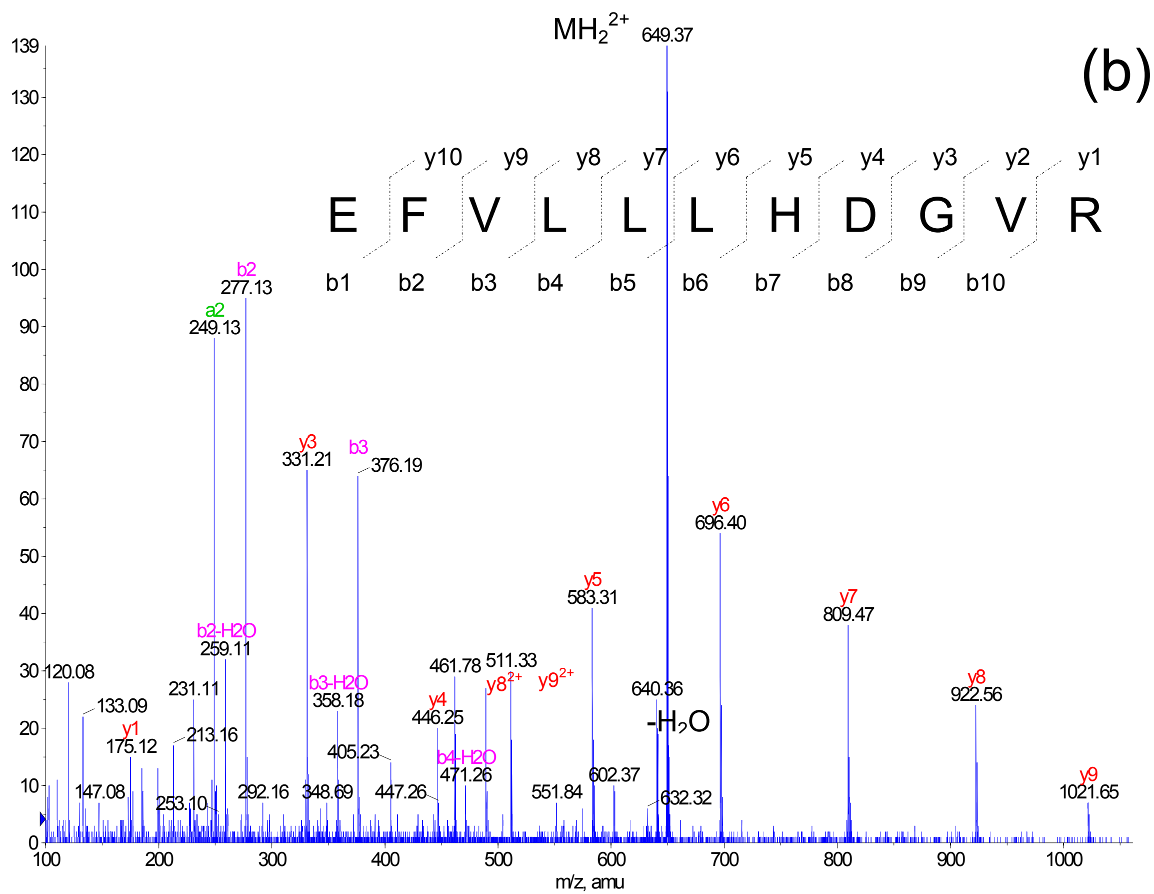


Figure 5.5, Continued (B).

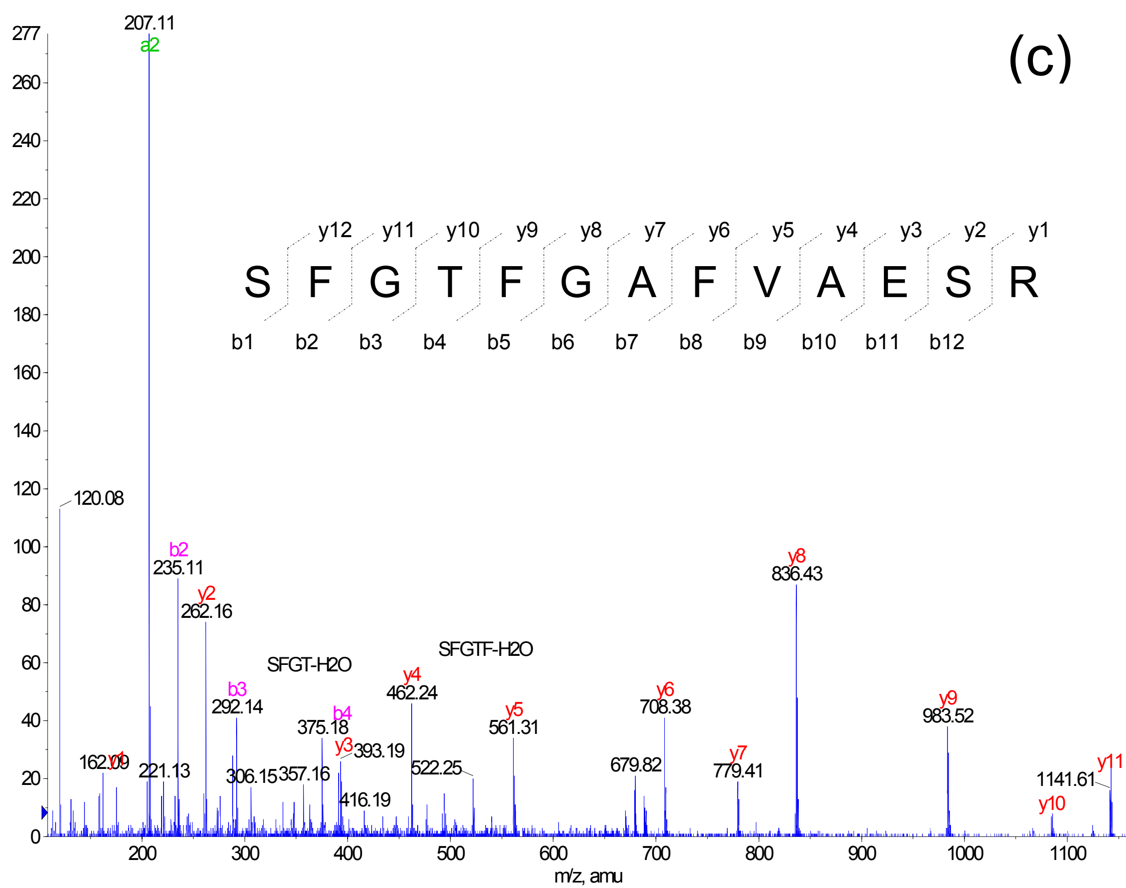


Figure 5.5, Continued (C).

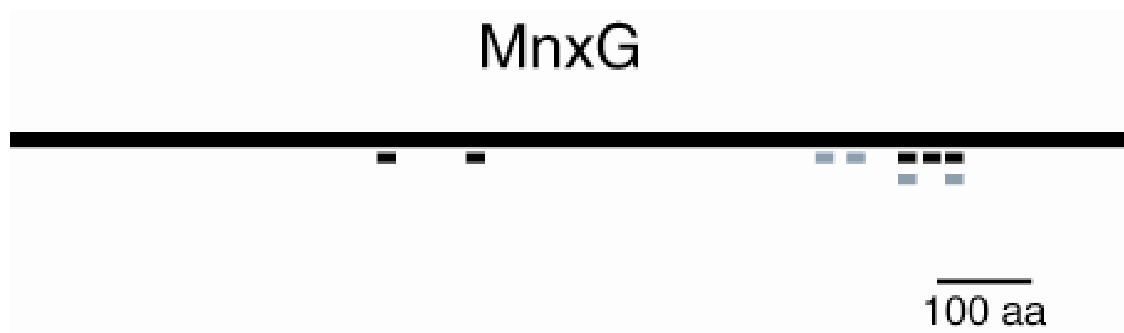


Figure 5.6. Schematic representation of the predicted MnxG protein (solid black line), showing location of peptides detected by MS/MS from PL-12 (black) and MB-7 (gray).

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VI

Genomic insights into Mn(II) oxidation by *Aurantimonas* sp. strain SI85-9A1, a globally distributed Mn(II)-oxidizing α-proteobacterium

Abstract

The Mn(II)-oxidizing α-proteobacterium SI85-9A1 has long been known to contain ribulose-1,5-bisphosphase carboxylase/oxygenase (RubisCO) genes for carbon fixation, but little is known about the mechanism of Mn(II) oxidation, the role of carbon fixation, or the potential link between the two. Here we report that SI85-9A1-like Mn(II)-oxidizers are present in diverse environments from around the globe. Whole genome DNA-DNA hybridization indicates that SI85-9A1 is a novel species of the genus *Aurantimonas*, and genome sequence provided insights into overall metabolism as well as the mechanism and physiology of Mn(II) oxidation. SI85-9A1 is metabolically versatile, with pathways for heterotrophy, methylotrophy, and lithotrophic growth on sulfur and carbon monoxide. The complete Calvin cycle for carbon fixation is present, suggesting that it is capable of autotrophic growth. A putative Mn(II) oxidase is encoded by duplicated multicopper oxidase (MCO) genes with homology and genomic context that suggest involvement in copper resistance. The Mn(II) oxidase is excreted to the extracellular medium along with an abundant hemolysin-type Ca^{2+}-binding peroxidase. Supplementation of heterotrophically growing cultures of SI85-9A1 with Mn(II) dramatically stimulates growth, but it is unclear if this stimulation is due to use of Mn(II) as an energy source or some other benefit of Mn(II) such as protection from reactive oxygen species.

Introduction

Mn(II)-oxidizing bacteria are thought to be responsible for catalyzing the formation of most naturally-occuring Mn oxides, minerals that are abundant in both terrestrial and marine environments (Tebo et al., 2004). As strong scavengers, Mn oxides control the distribution and availability of many trace elements, and as the strongest environmentally relevant oxidant after oxygen, they serve as electron acceptors for anaerobic respiration (Tebo et al., 2004). Because of this broad influence that Mn oxides exert on biogeochemical cycles, it is important to understand the biological mechanism of their formation. The ability to oxidize Mn(II) is widespread among phylogenetically diverse bacteria, however the biochemical mechanism is only beginning to be elucidated, and the physiological function of Mn(II) oxidation is unclear (Tebo et al., 2005). Mn(II) oxidation by O_2 is thermodynamically favorable and has long been hypothesized to support chemolithoautotrophic growth, but to date no link between autotrophy and Mn(II) oxidation has been clearly demonstrated.

The marine α-proteobacterium strain SI85-9A1 is perhaps the most compelling case for a Mn(II)-oxidizing autotroph because it has genes for ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Caspi et al., 1996), the key enzyme of carbon fixation by the Calvin-Benson cycle (Tabita, 1999). SI85-9A1 was isolated from the oxic/anoxic interface (100 m depth) of Saanich Inlet in 1985 based on its ability to oxidize Mn(II) to Mn(III/IV) oxides during heterotrophic growth (Tebo, unpublished). Though unable to grow with Mn(II) as the sole energy source under laboratory conditions, SI85-9A1 remains of interest because of the possibility that its' RubisCO is linked to Mn(II)

oxidation either under unknown conditions or during mixotrophic growth. The RubisCO gene shows CO_2-fixing activity when expressed in *E. coli*, but no RubisCO activity has been detected in cultures of SI85-9A1. Therefore the role of this RubisCO in carbon fixation and the possible link to Mn(II) oxidation is unclear (Caspi et al., 1996).

SI85-9A1 is one of a growing number of diverse Mn(II)-oxidizers within the α-proteobacteria, where mechanisms of Mn(II) oxidation have only recently been investigated (Francis et al., 2001; Hansel and Francis, 2006; Larsen et al., 1999). Biochemical evidence suggests that Mn(II) oxidation by two α-proteobacteria, strain SD-21 (Francis et al., 2001) and *Pedomicrobium* strain ACS 3067 (Larsen et al., 1999), is catalyzed by a multicopper oxidase enzyme as in other Mn(II)-oxidizing bacteria (Tebo et al., 2005). Still, the biochemical mechanism and physiological function of Mn(II) oxidation by α-proteobacteria, especially strain SI85-9A1, remain largely unexplored. In this study we present evidence that SI85-9A1-like Mn(II)-oxidizing bacteria are widely distributed in diverse marine environments, from surface waters of the Sargasso sea to surfaces of the deep-sea hydrothermal vent tube worm *Alvinella pompejana*. Genome sequence and biochemical characterization of SI85-9A1 provided insights into the mechanism and physiology of bacterial Mn(II) oxidation.

Materials and Methods

Isolation, identification, and characterization of Mn(II)-oxidizing SI85-9A1-like bacteria. *Alvinella pompejana* epibionts were isolated during the Extreme 2001 cruise (October/November, 2001) to the EPR aboard the *R/V Atlantis*. *A. pompejana*

worms were collected with *DSV Alvin* (dive 3713) from a depth of 2494 m at P-vent, located on the EPR at 9°N. Shipboard, dorsal hairs were removed from the worm using a sterile scapel, then washed and homogenized in filter-sterilized natural seawater. This homogenate was spread on J-media agar plates that contained no organic carbon source (Tebo et al., 2006) and incubated at 37°C. Brown, Mn oxide-encrusted colonies became apparent after 11 days and were transferred to J plates at room temperature. The presence of Mn oxide was confirmed with the leukoberbelin blue (LBB) spot test (Tebo et al., 2006). These isolates are typically maintained on M medium (Tebo et al., 2006) at room temperature. DNA extraction, PCR, and sequencing of 16S rRNA genes were as described previously (Francis and Tebo, 2002).

Bacteria with highly similar 16S rRNA gene sequences to SI85-9A1 were obtained from the following sources: *Aurantimonas coralicida* (Denner et al., 2003), L. Richardson; *F. pelagi* HTCC 2506, 2615, and 2619 (Cho and Giovannoni, 2003), S.J. Giovannoni; isolate HTCC 2156, J. Cho; isolate ARK 126 (Mergaert et al., 2001), BCCM/LMG Bacteria collection (LMG 23055); isolate Eplume 4 J1 (Kaye and Baross, 2000), J. Baross. Mn oxidation by these isolates was tested on J, M, and K plates and confirmed with the LBB spot test (Tebo et al., 2006). Whole-genome DNA-DNA hybridization was performed in 2 X SSC and 10% formamide at 69°C by the Deutshce Saamlung von Mikrooorganismen und Zellkulturen GmbH (DSMZ). Sequence alignment and phylogenetic analysis of 16S rRNA was done using MEGA3.1 (Kumar et al., 2004). Alignments were manually inspected and edited to remove gaps and ambiguously aligned regions. The 16S rRNA phylogeny was constructed using the neighbor-joining method and the Kimura two-parameter evolutionary model.

DNA extraction, cloning, genome sequencing, and annotation. DNA for genome sequencing was extracted with the Qiagen DNeasy Tissue Kit. Shotgun cloning and sequencing was performed by the J. Craig Venter Institute as part of the Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project (https://research.venterinstitute.org/moore/). Automated annotation and complete manual curation of the SI85-9A1 genome was done with the MAGPIE system (Gaasterland and Sensen, 1996).

Growth Experiments. All growth experiments were done with J medium (Tebo et al., 2006) supplemented with either methanol (05. to 2.5 % v/v), glycerol, succinate, acetate, formate, or glycerol (10 mM each). Where indicated, Mn was added as $MnCl_2$ to a final concentration of 100 μM. Optical density was measured at 600 nm. To avoid interference of Mn oxide, cultures containing Mn were reduced with 1 mM ascorbic acid prior to measurement of optical density. Oxidized Mn was quantified using the LBB method (Tebo et al., 2006).

Biochemical Analysis. Cultures for biochemical analysis were grown on either J medium (supplemented with 10mM succinate or glycerol) or M medium (Tebo et al., 2006). Cultures (500-ml to 1 L) were pelleted by centrifugation at 10,000g. Supernatant was concentrated (1 - 4 L) by ultrafiltration (50-kDa MWCO, Millipore). Cells were lysed by passage through a french pressure cell 4 times at 20,000 psi. Mn(II)-oxidizing activity was assayed by the LBB method (Tebo et al., 2006). SDS-PAGE (10%) analysis was done on concentrated supernatant from stationary phase cells grown on J-glycerol (without Mn added) as described previously (Francis and Tebo, 2002). After the in-gel

Mn(II)-oxidizing activity assay, the gel was submerged in LBB to identify the presence of a Mn oxide band.

In-gel trypsin digestion and LC-MS/MS. In-gel trypsin digestion of coomassie and in-gel Mn(II) oxidizing activity bands and LC-MS/MS was performed as described in Chapter V. MS/MS spectra were analyzed with Analyst QS 1.1/ProID 1.1 (Tang et al., 2005) and BioAnalyst 1.1.5/ProBlast 1.1 (Schevenko et al., 2001) (Applied Biosystems).

Results and Discussion

Isolation, Mn(II) oxidation, and phylogeny of SI85-9A1-like bacteria from diverse environments. We cultivated a number of Mn(II)-oxidizers from an *Alvinella pompejana* tube worm during the Extreme 2001 cruise to 9°N on the East Pacific Rise (EPR). *Alvinella pompejana* is a polychaete tube worm that inhabits the walls of black smoker chimneys at deep-sea hydrothermal vent sites along the EPR. This habitat lies at the interface of hot, reducing, metal-laden hydrothermal fluids and cold, oxygenated deep-sea waters. Previous studies noted the prevalence of Mn oxides (B. Glazer, personal communication) and Mn(II)-oxidizing bacteria (Prieur et al., 1990) in the dense microbial communities that inhabit the dorsal surface of *A. pompejana,* and hypothesized that these epibiotic bacteria might be involved in metal detoxification (Alayse-Danet et al., 1987). To identify these Mn(II)-oxidizing bacteria, five isolates were chosen for 16S rRNA gene sequencing. All five were found to have identical 16S rRNA sequence to SI85-9A1 (1400 bp considered).

Recently, the isolation of a number of other bacteria closely related to SI85-9A1 has been reported. These bacteria hail from diverse environments of distinct ocean basins, including surface waters of the Sargasso (F. pelagi HTCC2506 (Cho and Giovannoni, 2003), HTCC 2156 (J. Cho and S.J. Giovannoni, unpublished)), Greenland (ARK 126 (LMG 23055) (Mergaert et al., 2001)) and Mediterranean (strain V4.MO.18, C. Stroempl, unpublished) Seas; a coral from the Florida Keys (Denner et al., 2003), and a hydrothermal plume on the Juan de Fuca Ridge (Kaye and Baross, 2000). These isolates form a tight phylogenetic cluster, and four of the six tested are able to oxidize Mn(II) to Mn oxides (Fig. 6.1). Despite sharing nearly identical 16S rRNA sequences, whole genome DNA-DNA hybridization of SI85-9A1 to its closest relatives revealed very low similarity (Table 6.1), suggesting that SI85-9A1 represents a new species within the genus *Aurantimonas*.

General genome features of SI85-9A1. *Aurantimonas* sp. strain SI85-9A1 was chosen for genome sequencing, resulting in 4,325,257 bp of sequence that assembled into one major contig and 23 small fragments (41,471 bp total). A combination of automated and manual annotation predicted 3653 ORFs including 3 rRNA operons and 50 tRNA genes (Table 6.2). The majority of these genes had best BLAST hits to organisms of the genus *Mesorhizobium* (Table 6.2), the closest relatives of SI85-9A1 for which genome sequences are available (Fig. 6.1). Although the genome was not closed, the draft sequence we report here has proven to be an invaluable resource for mass spectrometry data analysis and has provided insights into metabolic capabilities as well as the mechanism and physiology of Mn(II) oxidation.

Metabolic versatility (i): Lithotrophy and methylotrophy. Strain SI85-9A1 was isolated as a heterotrophic bacterium capable of oxidizing Mn(II) while growing on organic carbon, therefore we were surprised to discover that the genome sequence encodes the genetic potential for growth on several inorganic substrates and one-carbon compounds. The complete set of genes for lithotrophic sulfur oxidation is present in an arrangement (*soxTRSVWXYZABCDEFGH*) identical to that found in the model sulfur oxidizer *Paracoccus pantotrophus* (Friedrich et al., 2005). Utilization of sulfur compounds could account for a substantial fraction of SI85-9A1's energy generation in its natural habitat, an oxic/anoxic interface where reduced sulfur species are abundant. Another potential source of inorganic energy is carbon monoxide; the 9A1 genome contains an operon encoding the large, medium, and small subunits of aerobic carbon monoxide dehydrogenase, and a second copy of the large subunit is present at a separate chromosomal location (Table 6.3).

The complete suite of genes required for the oxidation of methanol to carbon dioxide are present: methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase (Table 6.3). Putative genes for methanol sensing and regulation were also detected. No particulate or soluble methane monooxygenase genes were found. SI85-9A1 is able to grow weakly on methanol as the sole energy and carbon source, (e.g. final OD600 = 0.01), therefore carbon assimilation may occur via the energetically inefficient Calvin cycle as in some other facultative methylotrophs (Dedysh et al., 2005).

Metabolic versatility (ii): Heterotropy. In addition to being capable of growth using inorganic energy sources, SI85-9A1 is able to utilize a wide variety of organic carbon compounds, including organic acids (succinate, acetate), glycerol, and sugars

such as glucose. Its ability to lead a heterotrophic lifestyle is reflected in a large number of high affinity transporters (ABC-type and TRAP-type) for dicarboxylates, amino acids, peptides, and carbohydrates. Complete central pathways for glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate cycle could be reconstructed.

Metabolic versatility (iii): Carbon fixation. The complete Calvin-Benson-Bassham cycle for carbon fixation is encoded in an operon of genes *cbbRFPTALSXE* (Table 6.3), including the form I type C/D RubisCO that was previously cloned from SI85-9A1 (Caspi et al., 1996). This suggests that this SI85-9A1 RubisCO gene is involved in carbon fixation rather than being a relic of an autotrophic descendent (Caspi et al., 1996). The presence of the regulator cbbR is consistent with tight control of the *cbb* operon as in other facultative autotrophs that are metabolically versatile (Kusian and Bowien, 1997). A second copy of the large subunit of RubisCO is also present, as well as one copy of a RubisCO-like protein (Form IV) which does not function in carbon fixation but rather is thought to be involved either in sulfur metabolism, ROS defense, or biosynthesis (Ashida et al., 2005; Hanson and Tabita, 2001; Li et al., 2005).

Mn(II) oxidation and effect of Mn(II) on growth. Most Mn(II)-oxidizing bacteria that have been studied to date oxidize Mn(II) during late-exponential or stationary phase or after sporulation (Tebo et al., 2005). In contrast, strain SI85-9A1 oxidizes Mn(II) at an early stage of growth (Fig. 6.2), similar to *Pedomicrobium* sp. ACM 3067 (Larsen et al., 1999). Mn(II) oxidation takes place during growth on organic acids (formate, succinate, acetate) and glycerol but not during growth on glucose, despite the fact that growth is fastest on glucose. Growth on glycerol is extremely slow, and the presence of $MnCl_2$ increases the growth rate of strain SI85-9A1 (Fig. 6.2). An increase in

overall growth yield with Mn addition/oxidation has been observed in the Mn(II)-oxidizing α–proteobacterium strain SD-21 (Francis et al., 2001) and in *Pseudomonas putida* GB-1 (Parikh and Chorover, 2005), but the nature of this stimulation is unknown. Mn is a micronutrient for many enzymes but is not expected to limit growth under the non-trace metal clean conditions under which our experiments were performed. Mn could be providing protection from oxidative stress, which SI85-9A1 may be facing as discussed further below (see *Mechanism of Mn oxidation* section). Mn plays a prominent role in biology in protecting cells from reactive oxygen species (ROS) (Daly et al., 2004; Horsburgh et al., 2002), and Mn supplementation rescues ROS defense mutants in *Bacillus subtilis* and *Escherichia coli* (Al-Maghrebi et al., 2002; Inaoka et al., 1999). Finally, Mn(II) oxidation could be beneficial as an energy source by generating a proton motive force and/or driving ATP synthesis. SI85-9A1 has the metabolic pathways for carbon fixation via the Calvin cycle, but will not grow autotrophically with Mn(II) as the sole electron donor in our hands. Nevertheless, Mn(II) oxidation could benefit the cell as an energy supplement or by driving proton gradient-driven processes such as high-affinity transport or motility. Mn(II) oxidation could generate a proton motive force by several mechanisms. Mn(II)-derived electrons could be passed along an electron transport chain which would drive a proton gradient, consistent with various cytochromes being required for Mn(II) oxidation by several Mn(II)-oxidizing species (Caspi et al., 1998; Tebo et al., 1997b; Tebo et al., 2005). In addition, protons are a product of the Mn(II) oxidation reaction itself, therefore extracellular Mn(II) oxidation could serve to increase the extracellular proton concentration. Further physiology experiments are

underway to determine the nature of the stimulation of growth by Mn and whether energy is derived from Mn(II) oxidation by strain SI85-9A1.

Mechanism of Mn oxidation. Strain SI85-9A1 was assayed for Mn(II)-oxidizing activity in vitro. Mn(II)-oxidizing activity did not depend on the presence of Mn in the growth medium. The majority of Mn(II)-oxidizing activity was recovered from the growth media supernatant of pelleted cultures rather than from whole cells or lysed cell extracts, indicating that the Mn(II)-oxidizing enzyme is excreted. SDS-PAGE analysis of concentrated cell-free growth media revealed seven distinct coomassie stainable bands in this extracellular fraction (Fig. 6.3). In-gel tryptic digestion followed by nanoLC-MS/MS unambiguously identified 6 of these bands. Five bands (A-E) all belong to one large (predicted 350-kDa) protein that has hemolysin-type calcium-binding region signatures and belongs to the animal heme peroxidase superfamily (Pfam PF03098, PROSITE PDOC00394). A sixth band (F) was identified as a putative periplasmic substrate-binding protein from an ABC-type transporter, and the seventh band (G) contained peptides from two different proteins – the putative periplasmic substrate-binding protein as well as a hypothetical protein. SDS-PAGE gels of the extracellular protein fraction were also screened with an in-gel activity assay for Mn oxidation (Francis and Tebo, 2002), revealing one grey-colored ~50-kDa band. The composition of this band was confirmed to be Mn oxide with by immersing the gel in LBB, a colorometric indicator that reacts specifically with oxidized Mn to produce a cobalt blue color (Fig. 6.3) (Tebo et al., 2006). This in-gel Mn oxide formation indicates a Mn(II)-oxidizing enzyme of an apparent molecular weight of ~50-kDa. The grey color is unusual as all other in-gel Mn oxide bands identified to date are brown or orange

(Francis and Tebo, 2002); this unique color may indicate that the primary biogenic Mn oxide mineral produced by SI85-9A1 is distinct from the better studied orange/brown Mn oxides produced by *Bacillus* spores (Bargar et al., 2005; Webb et al., 2005b). A second light blue band was apparent at > 250 kDa, indicating the presence of oxidized Mn in the gel at that location as well. Since no Mn oxide band was visible at that spot prior to LBB staining, it is unclear whether this LBB band is indicative of a small quantity of Mn oxide or the presence of oxidized Mn(III) or (IV) in some other state (e.g. bound to a protein). No peptides were recovered from either LBB positive band by nanoLC-MS/MS, therefore their identity is uncertain. These results are consistent with the Mn(II) oxidase being present at very low abundance despite high Mn(II)-oxidizing activity, a characteristic observed in other Mn(II)-oxidizing bacteria (see Chapter V). It is difficult to determine whether the LBB-positive bands correspond to any of the coomassie bands because the coomassie-stained samples shown in Fig. 6.3 were boiled prior to SDS-PAGE (boiling was required to get well-defined bands), but samples for the in-gel activity assay were not boiled (because boiling eliminated activity).

All Mn(II)-oxidizing bacteria studied to date require multicopper oxidase (MCO) genes for Mn oxidation (Tebo et al., 2005), and the MCO MnxG was recently demonstrated to be the direct catalyst of Mn(II) oxidation by marine *Bacillus* spores (see Chapter V). MCOs utilize Cu as a cofactor in coupling four one-electron substrate oxidations to the four-electron reduction of O_2 to H_2O (Solomon et al., 1996). MCOs are a large family of enzymes with high sequence divergence, however the amino acids involved in coordinating Cu ions are highly conserved and easily recognizable. The SI85-9A1 genome contains three genes coding for proteins that contain the four Cu-

binding amino acid sequence motifs of MCOs (HXH, HXH, HXXHXH, and HCHXXXH (Solomon et al., 1996)) and a fourth gene that contains three of the four MCO motifs (missing HXXHXH). Two of the MCOs are nearly identical to each other (98% amino acid (AA)) and code for proteins that show significant homology (65% AA) to the amino sequence for MoxA, a MCO that was deposited in Genbank and annotated as essential for Mn(II) oxidation and laccase-like activity in the α-proteobacterium *Pedomicrobium* sp. ACM 3067 (CAJ19378). The predicted 51-kDa size of the MoxA-like proteins (referred to hereafter as MoxA-1 and MoxA-2) matches the size of the Mn(II)-oxidizing enzyme identified by the in-gel Mn(II)-oxidizing activity assay (Fig. 6.3). Based on their similarity to MoxA and the fact that they are predicted to be the same size as the Mn(II) oxidase, we suspect that MoxA-1/2 are the Mn(II)-oxidizing enzymes. Both MoxA-like proteins have twin-arginine signal peptides that are similar to the TAT secretory pathway consensus sequence (Berks et al., 2000). Although the TAT system usually targets proteins to the periplasm, TAT proteins can be subsequently exported across the outer membrane by the general secretory pathway (Gsp) (Voulhoux et al., 2001) as would be expected based on the extracellular Mn(II)-oxidizing activity. In fact, a novel Gsp-type pathway is required for export of the Mn(II)-oxidizing enzyme across the outer membrane of *Pseudomonas putida* strain GB-1 (de Vrind et al., 2003). An operon encoding the Gsp machinery (ZP_01225900 to ZP_01225907) is present in SI85-9A1, suggesting a similar export system for the Mn(II)-oxidizing system in this organism. Interestingly, this Gsp operon is adjacent to the gene for the excreted Ca^{2+}-binding peroxidase that may be involved in the Mn(II) oxidation system as discussed further below. MoxA-1/2 each contain two methionine-rich regions following two of the Cu-

binding regions. Methionine rich regions have been hypothesized to play a role in binding extra Cu ions (Mellano and Cooksey, 1988) that are thought to play a regulatory role in the *E. coli* MCO CueO (Roberts et al., 2003).

Homologs of MoxA are found in many α-proteobacterial genomes, but experimental data on the function of these genes is only available from a few distantly related MCOs (Table 6.4). The closest relative of MoxA-1/2 (60% aa identity) is a protein (ORF4) that was recently discovered based on its involvement in Cu resistance in the plant pathogen *Xanthomonas campestris* pv. vesicatoria (Basim et al., 2005). The *orf4* gene is induced by Cu, and is encoded on the chromosome in an operon of Cu-resistance genes that also share similarity with MoxA neighbors (discussed further below). Other MoxA-1/2 homologs that have been experimentally associated with a function are more distantly related (Table 6.4). Typically they are involved in Cu resistance, although as pointed out by Huston (Huston et al., 2002), in many cases the resistance conferred is only marginal. This invites speculation that the Cu sensitive phenotype is due to indirect effects, and that the MCO may in fact have other physiological functions. Indeed these enzymes often exhibit broad specificity, oxidizing substrates such as iron (Huston et al., 2002), Cu (Singh et al., 2004), and organics including siderophores (Grass et al., 2004). Therefore it has been difficult to discern whether phenotypes of MCO mutants are due to a direct role in Cu homeostasis or because of loss of some other function, such as iron uptake (as demonstrated by Huston et al) or siderophore oxidation. Neither the *Pseudomonas aeruginosa* MCO nor CueO are able to oxidize Mn(II) (Huston et al., 2002; Kim et al., 2001), so MoxA may represent a new class of bacterial MCO that is specific (at least in terms of metals) for Mn(II).

MoxA-1 and *moxA-2* occur in nearly identical three-gene operons that are located at two separate chromosomal sites. In both operons, upstream of *moxA* there are genes coding for predicted proteins that are similar to MoxB (40% AA), which is also in the *moxA* operon of *Pedomicrobium* sp. ACM 3067 (CAJ19377). MoxB shows some similarity to the TolC family of proteins that are involved in Type 1 secretion, but the TolC system is involved in efflux of many types of molecules, including AQDS, antibiotics, detergents, dyes and phenazines ((Shyu et al., 2002) and references therin). Strain SI85-9A1's MoxB also shares 31% amino acid identify with ORF3 from *Xanthomonas campestris* pv. vesicatoria (AAP42070), where it is involved in Cu resistance and is induced by low levels of Cu (Basim et al., 2005). Therefore a number of physiological roles for MoxB seem feasible. Downstream of *moxA* is a gene encoding a small predicted protein (~18-kDa) with similarity to COG4454, an uncharacterized copper-binding protein. This protein is 47% similar (amino acid identity) to ORF5 of *Xanthomonas campestris* pv. vesicatoria (AAP42068), also thought to play a role in Cu resistance (Basim et al., 2005). The location of the *moxA* gene in an operon of genes that appear to be involved in Cu resistance may indicate that this putative Mn(II) oxidase also plays a role in Cu resistance. Alternatively, the connection between the MCO and the Cu homeostasis genes could arise from the MCO's requirement for Cu as a cofactor since these Cu resistance genes could actually be involved in Cu acquisition or sequestration.

The putative Mn(II) oxidase operons are part of two larger 15 and 19-kb regions with at least eight genes that share significant sequence similarity to each other (64 – 98 %), suggesting the entire region has been duplicated. Included in this region are a number of interesting genes related to trace metal homeostasis and possibly Mn(II)

oxidation. Near the Mox-1 region there is a predicted heavy metal transcriptional regulator in close proximity to MerT (mercury transport protein) and MerA (mercury reductase), as well as a MCO-like protein that has some sequence similarity to MoxA, but lacks one of the Cu-binding regions. In the Mox-2 region there is a permease of the major facilitator family predicted to be a nickel resistance protein. Here there is also a putative sensory histidine protein kinase and a two-component response regulator that show some limited similarity to the CopR/PcoR and CopS/PcoS proteins, respectively, that are involved in regulating Cu resistance systems (Mills et al., 1993; Munson et al., 2000). The proteins that are duplicated in both Mox regions include several hypothetical and conserved hypotheticals, as well as a cytochrome C. Cytochromes are required for Mn(II) oxidation in several Mn(II)-oxidizing bacteria (Tebo et al., 2005), and it is tempting to speculate that the presence of a cytochrome C in close proximity to the Mn(II)-oxidizing system reflects a connection between Mn(II) derived electrons and an electron transport chain.

An excreted Ca^{2+}–binding peroxidase (ECP). The function of the excreted hemolysin-type Ca^{2+}-binding peroxidase identified by MS/MS is unclear, but several features are of interest as they may relate to Mn(II) oxidation. The protein contains 18 G-G-x-G-x-D motifs (and 10 of the more specific consensus Ca^{2+}-binding hemolysin pattern D-x -[LI]-x(4)-G-x-D-x-[LI]-x-G-G-x(3)-D used by PROSITE) that target proteins for export by the Type I secretion system (Duong et al., 1994; Letoffe and Wandersman, 1992) and have been shown to bind Ca^{2+} ions (Baumann et al., 1993). This pattern is found in excreted proteins ranging from toxins such as hemolysin in pathogenic bacteria to the nodulation protein NodO, thought to be involved in interactions between

rhizobia and their host plants (Economou et al., 1990). The Ca^{2+}-binding aspect is notable in light of connections between Ca^{2+} and Mn(II) oxidation that have recently emerged from several independent lines of evidence. Biogenic Mn oxides have a high affinity for Ca^{2+} (but not other divalent cations like Mg^{2+}), and Ca^{2+} is a common interlayer cation in biogenic Mn oxides (Webb et al., 2005b). Ca^{2+} was also recently shown to stimulate Mn(II) oxidation by *Bacillus* spores (K. Toyoda and B.M.T., in preparation) and by the α-proteobacterium SD-21 (H.A. Johnson and B.M.T., in preparation). Ca^{2+} is known to stabilize pyrrolo-quinoline quinone (PQQ), a cofactor associated with alchohol and amine dehydrogenases (Anthony, 1996) that plays a role in Mn(II) oxidation by the α-proteobacterium SD-21 (H.A. Johnson and B.M.T., unpublished). The SI85-9A1 genome contains an operon of three PQQ biosynthesis genes, but the role of PQQ in Mn(II) oxidation by SI85-9A1 has not been investigated.

ECP is also is a member of the animal heme peroxidases, a superfamily of proteins that is found throughout the proteobacteria and is especially common in the order Rhizobiales (to which strain SI85-9A1 belongs); however little is known about the function of these proteins in bacteria. Abundant excretion of the peroxidase may reflect oxidative stress, a cellular challenge in which Mn plays a prominent protective role (Horsburgh et al., 2002). Mn protects cells from reactive oxygen species both as a cofactor in enzymes such as Mn peroxidase and superoxide dismutase, and as a chemical reductant that is accumulated to high intracellular concentrations (Daly et al., 2004; Horsburgh et al., 2002). Mn(II) reduces superoxide to H_2O_2 (Archibald and Roy, 1992), which could then serve as a substrate for the peroxidase. One predicted peroxidase has even been shown to be involved in Cu resistance, likely by preventing the formation of

ROS that would result from reaction of Cu with hydrogen peroxide (Gupta et al., 1997). Thus there are potential physiological connections between the excreted peroxidase, which may be involved in ROS detoxification, and the putative Mn(II) oxidase, which is related to proteins involved in Cu resistance. Such a protective role may account for the increased growth rates of SI85-9A1 in the presence of Mn. Alternatively, the peroxidase may function in extracellular oxidation reactions. In fungi, Mn(II) serves as the reducing substrate for Mn peroxidase, which oxidizes Mn(II) to Mn(III) in the presence of organic chelators (Wariishi et al., 1992). There is also known connection between a Mn(II)-oxidizing laccase and Mn peroxidase in fungal systems; laccase enzymatically produces Mn(III) which leads to the abiotic generation of H_2O_2. This H_2O_2 then serves as the electron acceptor for Mn peroxidase mediated oxidation reactions of lignin and xenobiotics (Schlosser and Höfer, 2002). The co-occurence of a laccase-like Mn(II) oxidase and a peroxidase in the growth medium of SI85-9A1 cultures suggests that a similar process may be at work. To our knowledge, Mn peroxidase has not yet been described in bacteria.

Trace Metal homeostasis. As discussed above, the effect of Mn on growth, the genomic context of the putative Mn(II) oxidase gene, and the co-localization of the Mn(II) oxidase and an abundant peroxidase in the extracellular fraction all hint at connections between Mn(II) oxidation, trace metal homeostasis, and ROS defense. These connections are perhaps not surprising given the overlap of regulatory systems for trace metal homeostasis and ROS defense in bacteria that has been recognized in recent years (Horsburgh et al., 2002). In addition, the putative Mn(II) oxidase is a MCO, a family of enzymes known to be involved in Fe (Huston et al., 2002) and Cu homeostasis (Rensing

and Grass, 2003; Singh et al., 2004), and siderophore degradation (Grass et al., 2004). More direct connections between Mn(II) oxidation and trace metal homeostasis have emerged recently. Mn(III), an intermediate of Mn(II) oxidation (Webb et al., 2005a), is bound by siderophores with slightly greater affinity than their intended target, Fe(III), and thus may influence Fe bioavailability (Duckworth and Sposito, 2005; Parker et al., 2004). Conversely, Mn(II) oxidation appears to be regulated in some cases by the presence of other metals (R. Verity and B.M.T., unpublished). Thus it seems likely that the regulation and physiology of Mn(II) oxidation are interconnected with the complex cellular circuitry for metal homeostasis.

The SI85-9A1 genome contains three genes of the ferric uptake regulator (Fur) family. One of these is most similar to Fur homologs (ZP_01228946) recently found to function in Mn uptake regulation (Mur) rather than Fe uptake regulation in α-proteobacteria (Bellini and Hemmings, 2006; Diaz-Mireles et al., 2005; Platero et al., 2004). The major targets of this regulator, the *sitABCD* genes, encode a Mn^{2+}-specific ABC-type transporter that is present in the SI85-9A1 genome (ZP_01226419 to ZP_01226422). A second Fur family protein (ZP_01225776) is a putative Zn uptake regulator (Zur) that occurs immediately upstream of a putative ABC-type Zn transporter system, and the third Fur family protein (ZP_01228417) clusters with iron response regulator (Irr) proteins that are involved in heme biosynthesis (Platero et al., 2004). SI85-9A1 also has two RirA-like proteins, additional Fe-responsive regulators that do not have any sequence similarity to Fur (Todd et al., 2005). Overall, this complex regulatory system likely reflects the physiological need for a delicate balance between the intracellular levels of Fe, which satisfies the demand of Fe-proteins but brings risk of

ROS toxicity, and Mn, which plays a crucial role in defense against ROS. The regulatory players in our marine Mn(II) oxidizer appear to be similar to its terrestrial rhizobial relatives *Rhizobium leguminosarum* (Bellini and Hemmings, 2006; Diaz-Mireles et al., 2005) and *Sinorhizobium meliloti* (Platero et al., 2004), for which the view of metal regulation is dynamic but clearly distinct from the better known *Escherichia coli* and *Pseudomonas aeruginosa* systems. These N_2 fixing rhizobia require large amounts of Fe to satisfy the demands of nitrogenase; although SI85-9A1 does not have nitrogenase, apparently it retains similar mechanisms for acquiring iron and dealing with ROS toxicity.

SI58-9A1 has genes coding for high-affinity siderophore-mediated ferric iron uptake systems. There are three siderophore receptors, three ABC-type siderophore transporters, two other ABC-type transporters predicted to be specific for iron, and one gene for a TonB energy transducer. One four-gene cluster (ZP_01226434 to ZP_01226437) codes for siderophore biosynthesis and is most similar to that of *Rhodopseudomonas palustris*, which is predicted to encode a Rhizobactin-like siderophore (Larimer et al., 2004). It will be interesting to determine the Mn(III)-binding properties of this siderophore to determine whether it has high affinity for Mn(III) as in other Mn(II)-oxidizing bacteria (Duckworth and Sposito, 2005; Parker et al., 2004).

Six genes encoding CopA-type ATPases – proteins that drive the influx/efflux of metals – were detected. Two of these are predicted to be Cu-binding (ZP_01228072, ZP_01227337), and two others are adjacent to the Mox regions (ZP_01227209, ZP_01225535), perhaps suggesting that they too are involved in Cu transport. Interestingly, one of the CopA homologs (ZP_01227337) is predicted by Darkhorse (S.

Podell, submitted) to have been acquired by horizontal gene transfer, being most closely related to the archaeon *Methanosarcina barkeri* str. Fusaro. This putative inter-domain transfer is apparently unusual; in a recent study of the evolutionary history of CopA-type ATPases, only one case of likely inter-domain transfer was noted (Coombs and Barkay, 2005).

Conclusions. SI85-9A1-like Mn(II)-oxidizing bacteria are globally distributed in diverse environments. The genome sequence of SI85-9A1 reveals great metabolic versatility, with genetic potential for chemolithotrophic growth on reduced sulfur compounds, carbon monoxide, and methanol, and heterotrophic growth on a wide range of organic compounds. Unlike *Silicibacter pomeroyi*, where oxidation of reduced inorganic compounds is thought to provide an energy supplement that allows greater heterotrophic efficiency ("lithoheterotrophy", (Moran et al., 2004)), SI85-9A1 is capable of linking chemolithotrophy to carbon fixation via the Calvin cycle. This is the first example of a Mn(II) oxidizer that can grow chemolithoautotrophically (albeit not yet proven for Mn(II)). If Mn(II) oxidizers in nature are similarly metabolically versatile, fluxes of methanol, reduced sulfur species, or carbon monoxide may stimulate the activities of these organisms and therefore stimulate Mn(II) oxidation rates at oxic/anoxic interfaces where Mn(II) oxidation is prevalent (e.g. see Chapter II). Although we have demonstrated that Mn(II) increases the growth yield of SI85-9A1, it is unclear whether this reflects mixotrophic growth with Mn(II) serving as an energy source, or some other benefit such as protection from ROS. Thus the link between Mn(II) oxidation and carbon fixation remains in question. Further work including physiology and genome-wide gene

expression analysis under various growth conditions are required to determine the physiological function of Mn(II) oxidation.

Our results identified two nearly identical copies of an MCO gene that putatively encodes an excreted Mn(II) oxidase. The genomic context of these genes and the co-excretion of an abundant peroxidase hint at links between Mn(II) oxidation, trace metal homeostasis, and ROS defense. We hypothesize that the putative Mn(II) oxidases catalyze the laccase-like oxidation of Mn(II) to Mn(III), but the function of the duplicate copies and their role in the oxidation of Mn(III) to Mn(IV) are unclear at this time.
A survey of completed and ongoing microbial sequencing genome projects revealed that *moxA*-like genes are widespread throughout the α- (17/86 genomes), β- (21/53), γ- (1/159), and δ-proteobacteria (2/19). The distribution of these *moxA*-like genes is extremely scattered; in genera and species for which multiple genome sequences are available such as *Burkholderia* or *Rhodopseudomonas palustris*, *moxA* is often found in some strains/species but not others. If *moxA* is indeed a genetic marker for the ability to oxidize Mn(II), this spotty distribution could explain the polyphyletic nature of Mn(II)-oxidizing bacteria (Tebo et al., 2004; Tebo et al., 1997a; Tebo et al., 2005). m*oxA*-like sequences are also present in the metagenomic environmental datasets including the Sargasso Sea (Venter et al., 2004) and Annamox metagenome (Strous et al., 2006), indicating that they are abundant in the environment. The widespread occurrence of these genes suggests that they serve crucial physiological functions, and may indicate that bacterial Mn(II) oxidation is a more common trait than is currently recognized.

Table 6.1. Whole genome DNA-DNA similarity

	% Similarity (duplicate measurements)	
	SI85-9A1	HTCC 2156
A. coralicida	19.8, 23.8	5.9, 13.0
HTCC 2156	56.0, 56.8	x

Table 6.2. General Genome Features

Feature	Value
Total # coding sequences	3653
Similar to proteins of known function	2804 (77%)
Conserved hypothetical proteins	565 (15%)
Hypothetical proteins	284 (8%)
# rRNA operons	3
#tRNA genes	50
%GC content	67

Best BLAST hit genus	Number of genes
Mesorhizobium	1779 (49%)
Sinorhizobium	257 (7%)
Rhodopseudomonas	194 (5%)
Rhodobacterales	194 (5%)
Agrobacterium	108 (3%)
Bradyrhizobium	99 (3%)

Table 6.3. Genetic basis for metabolic versatility

Process		Protein(s)	Genbank Accession
Lithtrophy	Sulfur oxidation	Sox T/R/S/V/W/X/Y/Z/ A/B/C/D/E/F/G/H	ZP_01225759 to ZP_01225774
	CO oxidation	Aerobic carbon monoxide dehydrogenase	ZP_01227909 to ZP_01227911, ZP_01228739
Methylotrophy	Methanol sensing	MoxY	ZP_01228286
	Methanol regulation	MoxX	ZP_01228287
	Methanol oxidation	Methanol dehydrogenase	ZP_01228289 to ZP_01228291, ZP_01228705
	Formaldehyde oxidation	Formaldehyde dehydrogenase	ZP_01228291, ZP_01228274, ZP_01225718, ZP_01227698
	Formate oxidation	Formate dehydrogenase	ZP_01228971, ZP_01226368, ZP_01226369, ZP_01226481, ZP_01226482
Carbon Fixation	Calvin cycle	CbbR/F/P/T/A/L/S/X/E	ZP_01227925 to ZP_01227933
	Calvin cycle?	RubisCO, large subunit	ZP_01226398
	Unknown	RubisCO-like protein	ZP_01229072

Table 6.4. MoxA Homologs for which there is experimental data available

Protein	Accession	Organism	% AA identity	Function	reference
MoxA-1	ZP_01227197	*Aurantimonas* strain SI85-9A1	100	Mn oxidation?	This study
MoxA-2	ZP_01225555	*Aurantimonas* strain SI85-9A1	98	Mn oxidation?	This study
MoxA	CAJ19378	*Pedomicrobium* sp. ACM 3067	65	Mn oxidation	Ridge et al., in review
ORF 4	AAP42069	*Xanthomonas campestris* pv. Vesicatoria	60	Cu resistance	(Basim et al., 2005)
PcoA	Q47452	*E. coli*	24	Cu resistance	(Brown et al., 1995)
Pmco	AAN52530	*Pseudomonas aeruginosa*	24	Fe(II) uptake	(Huston et al., 2002)
CopA (ORF1?)	AAA72013	*Xanthomonas campestris pv. Juglandis*	23	Cu resistance	(Lee et al., 1994)
CueO	P36649	*E. coli*	23	Cu resistance	(Singh et al., 2004)
CopA	P12374	*Pseudomonas syringae*	23	Cu resistance	(Mellano and Cooksey, 1988)
CopA	AAF32269	*Aeromonas veronii* bv. sobria	23	slightly increased Cu tolerance	(Francki et al., 2000)

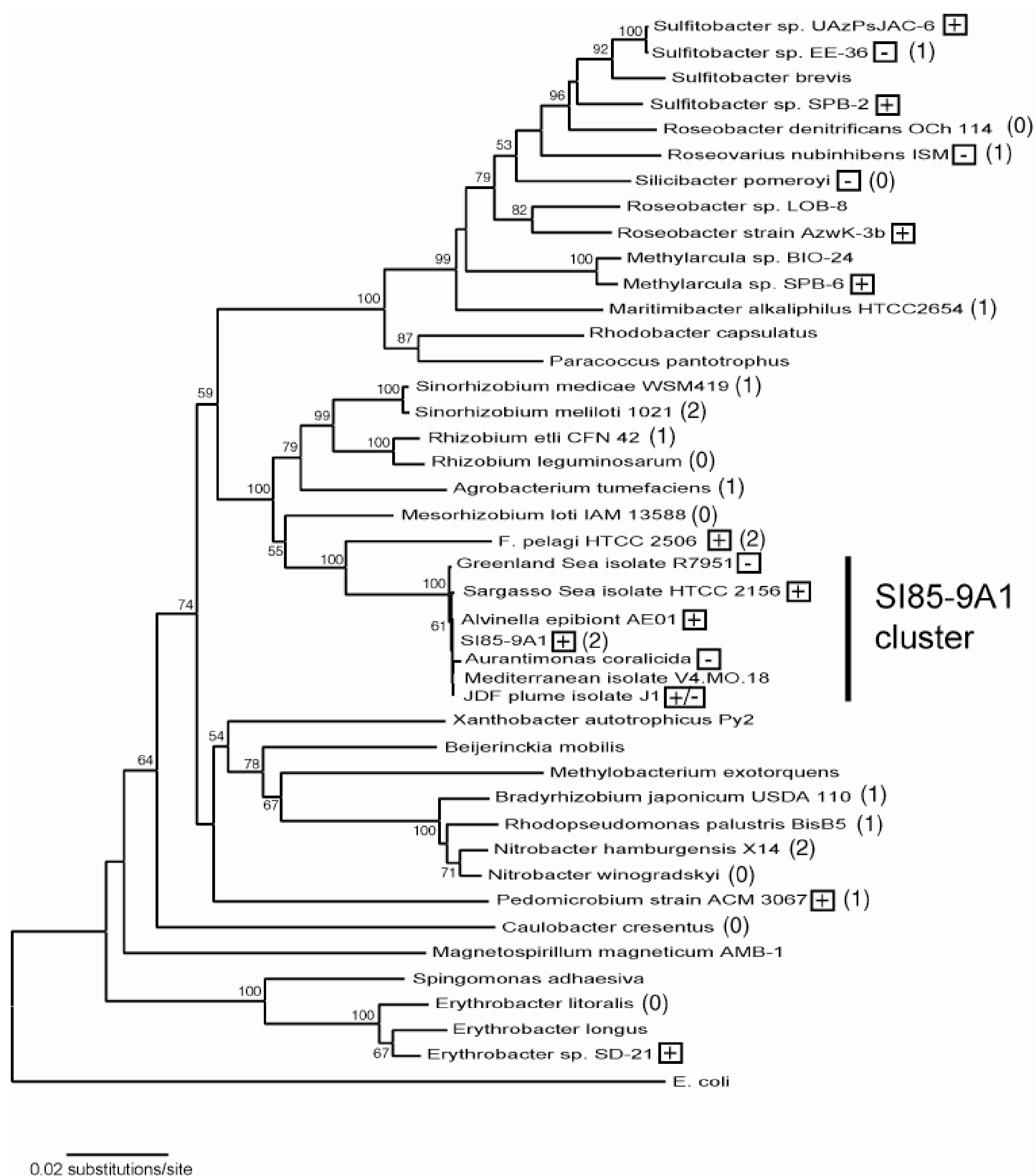

Figure 6.1. Phylogeny of Mn(II) oxidizing α-proteobacteria based on the16S rRNA gene. Mn(II) oxidizers are indicated with "+", isolates that have been tested and do not oxidize Mn(II) are indicated with "-", and "+/-" indicates weak or inconsistent Mn(II) oxidation. For organisms with genome sequences available, the number of copies of the *moxA* gene is indicated in parentheses.

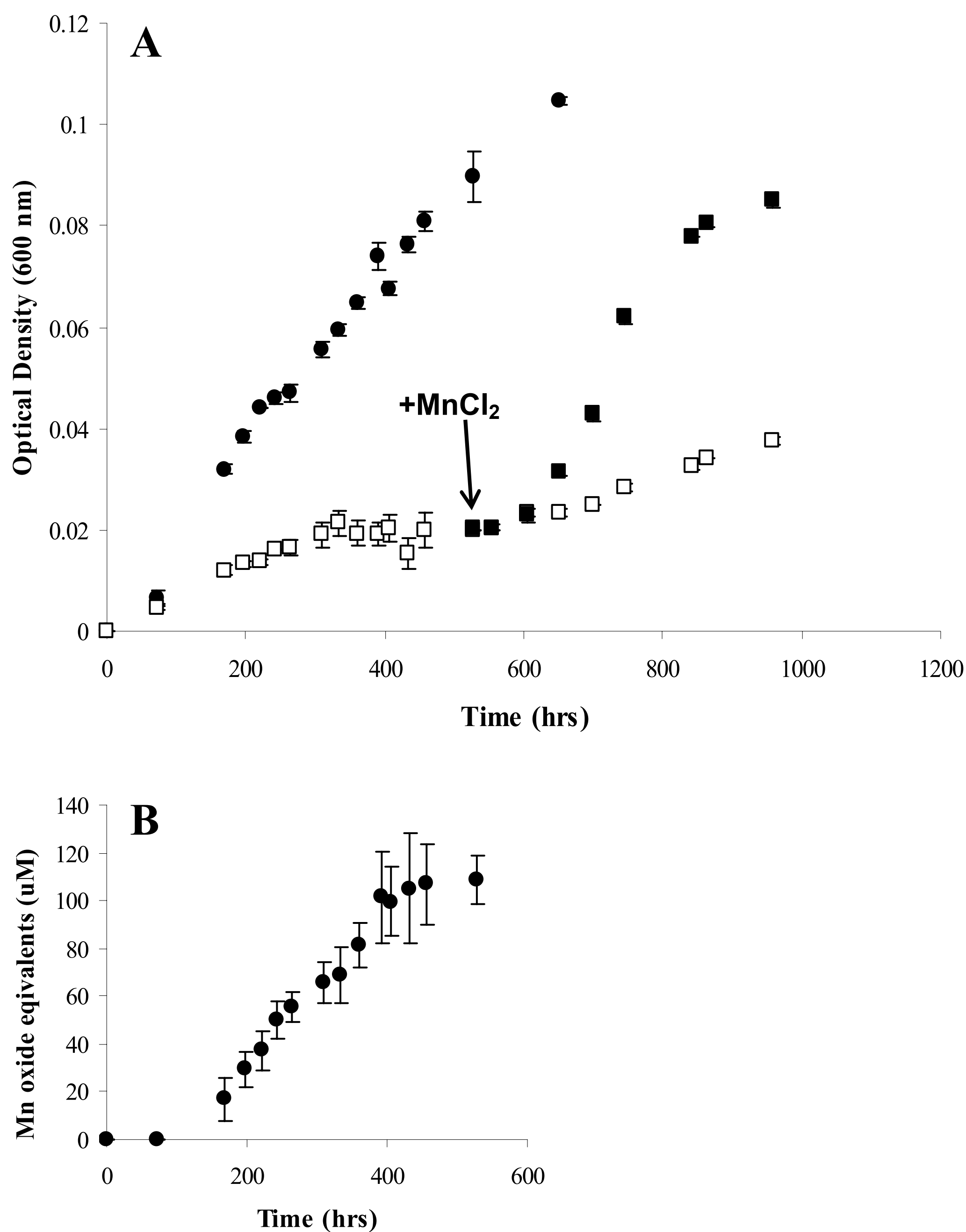

Figure 6.2. (A) Growth of SI85-9A1 on glycerol with 100 µM $MnCl_2$ (closed circles), without added Mn (open squares), and with 100 µM $MnCl_2$ added to replicates of the no Mn added culture in the middle of the growth curve (closed squares). (B) Mn oxide formation by the glycerol plus $MnCl_2$ culture shown in (A). Error bars represent standard deviation of triplicate cultures.

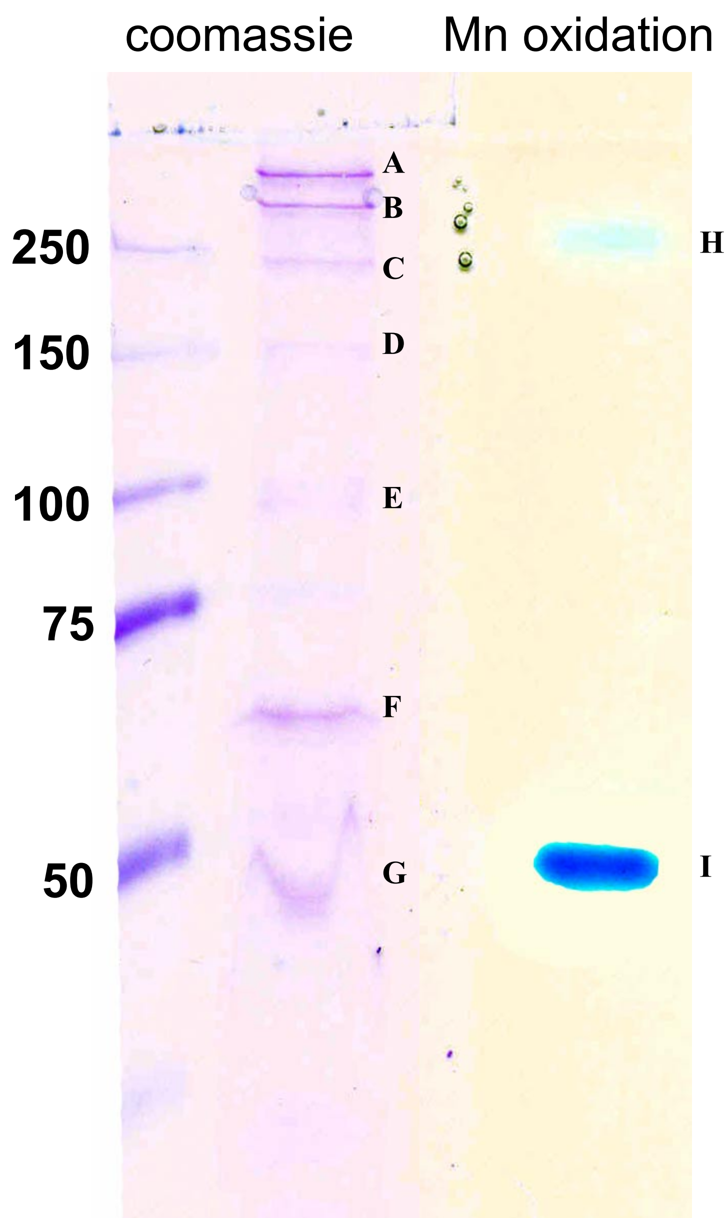

Figure 6.3. SDS-PAGE analysis of the SI85-9A1 extracellular fraction during growth on glycerol. The gel was cut in half and one half stained with coomassie (left) and one half screened with an in-gel Mn(II) oxidation activity assay (right). The latter was then immersed in LBB, which reacts specifically with Mn oxide to produce a cobalt blue color (H and I).

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VII

Conclusions and Future Directions

In this section I draw some broader conclusions and look toward the future to what I think are the most exciting directions for this field. This dissertation has applied a variety of approaches to address some of the long-standing questions regarding microbial Mn(II) oxidation, namely determining which microorganisms are responsible for Mn(II) oxidation in the environment, and the molecular and biochemical mechanisms by which Mn(II) oxidation is catalyzed. **Chapter III** set the stage for the environmental component of my work by identifying the Guaymas Basin (GB) deep-sea hydrothermal plume as a natural system where microbial enzymes drive the oxidative segment of the Mn cycle. **Chapters II and IV** included description of newly cultured Mn(II) oxidizers, however **Chapter IV** also revealed that the microbial community in the GB plume is dominated by microbes that are not currently known to oxidize Mn(II). This result suggests that the microbes that drive Mn(II) oxidation in nature have likely not yet been identified, nor have the factors controlling Mn(II) oxidation rates in the environment. In **Chapter V** the first direct identification of a Mn(II)-oxidizing enzyme was described, confirming the long held hypothesis that multicopper oxidases are the direct catalysts of Mn(II) oxidation. Finally, **Chapter VI** described the isolation and identification of Mn(II)-oxidizing organisms that have identical 16S rRNA sequences and are globally distributed. Analysis of the genome sequence of a representative of this group revealed that it is metabolically versatile and combined with preliminary biochemical characterization elucidated the likely molecular and biochemical mechanisms of Mn(II) oxidation.

Certainly one of the most interesting conclusions that can be drawn from this dissertation is the importance of chemical and nutrient conditions in regulation Mn(II) oxidizing genes and enzymes. In Chapter IV, this conclusion was drawn from environmental data; despite dramatic differences in Mn(II) oxidation rates between a plume and background deep-seawater, microbial communities in the samples differ only slightly, to an extent that cannot explain the observed biogeochemical data. The most likely explanation for this discrepancy is that rapid, plume-specific Mn(II) oxidation rates are the result of the unique activities of plume microbes rather than unique types of plume microbes. This notion is consistent with observations made on a Mn(II)-oxidizer in culture, where genomic insights suggest that the α-proteobacterium strain SI85-9A1 is metabolically versatile, being capable of growing heterotrophically or autotrophically on reduced sulfur compounds, carbon monoxide, or methanol. It has long been recognized that Mn(II) oxidation by this organism depends on the carbon source on which it is growing; it does not oxidize Mn(II) during growth on glucose (on which it grows best), but it does oxidize Mn(II) during growth on glycerol or organic acids. Preliminary work revealed that SI85-9A1 does oxidize Mn during autotrophic growth on thiosulfate (where it barely grows, but Mn oxides are apparent) and methanol. Clearly understanding the regulation of Mn(II) oxidation should be studied further, since stimulation of Mn(II) oxidation during chemolithoautotrophic growth could have far-reaching implications for why Mn(II) oxidation rates are rapid in environments such as deep-sea hydrothermal plumes and oxic/anoxic interfaces. Two obvious approaches to addressing this questions are (1) microarray analysis of genome-wide gene expression during different growth conditions, and (2) RNA-based studies of microbial activities as they occur in

environments such as the GB deep-sea hydrothermal plume. Development of a genetic system for SI85-9A1 would also be extremely valuable in understanding Mn(II) oxidation by this interesting organism. Although previous attempts in this area have been made (Caspi, 1996), advances in genetics of α-proteobacteria since then suggest that this problem should be revisited (Boscari et al., 2004; House et al., 2004; Wiedemann and Muller, 2004).

Genomic insights into Mn(II) oxidation by SI85-9A1 included hints that Mn(II) oxidation is linked to trace metal homeostasis (e.g. Cu resistance) and reactive oxygen species (ROS) defense. More physiological work is required to test these hypotheses. Although a putative Mn(II) oxidase was identified, that *moxA* encodes the Mn(II) oxidase needs to be confirmed. Better biochemical studies are required to determine whether the Mn(II) oxidase is really excreted, or whether it is loosely associated with the cell wall. Although the fact that Mn(II)-oxidizing activity is in the spent medium may make biochemical purification possible, the apparently low abundance of this enzyme may demand enormous scaling up of growth and purification procedures. Heterologous expression of *moxA* may provide a better means of obtaining large quantities of pure protein.

An astonishing insight gained by the linking of *moxA* to Mn(II) oxidation is that this gene is widespread in the genomes of organisms that have been sequenced and also in environmental datasets such as the Sargasso Sea (Venter et al., 2004). This has potentially profound implications in terms of the importance of Mn(II) oxidation in previously unrecognized organisms and environments such as terrestrial rhizobia, which are agriculturally important. This potential underscores the need for this link to be

confirmed, and for a better understanding of the biochemistry and physiology of Mn(II) oxidation by SI85-9A1. There should also be an investigation of whether *moxA* is a genetic marker for Mn(II) oxidation in diverse bacteria. So far, no Mn(II)-oxidizing α-proteobacteria are known to be missing *moxA*. While there are organisms with *moxA* that do not appear to be able to oxidize Mn, this could be due to loss of the ability to oxidize Mn during transfer in culture, which is not uncommon.

While this dissertation finally proved that a multicopper oxidase directly oxidizes Mn, the Mn(II) oxidizing enzyme has still not been fully purified and thus much remains to be learned about the biochemistry of Mn(II) oxidation. The low abundance of Mn(II) oxidases suggests that heterologous systems should be optimized for overexpression of Mn(II) oxidizing enzyme. Obtaining pure Mn(II) oxidase would allow a host of new lines of inquiry, especially looking at the specificity of Mn(II) oxidases (for Mn versus Cu, Fe, and organics) and the number and nature of Cu ion cofactors. These studies will be particularly intriguing for the *Bacillus* MnxG protein, for which Chapter V reported unusual Cu-binding signatures and catalysis of a two-electron transfer that is novel for multicopper oxidases. Further downstream approaches should include site-directed mutagenesis to determine the function of each Cu ligand, and determination of the crystal structure, which would allow a new set of structural/functional studies. Finally, pure Mn(II)-oxidizing enzyme would open up a new field of geomicrobiological research that could analyze the primary biogenic minerals that are the product of the Mn(II)-oxidizing enzyme. While such studies are ongoing on crude cell preparation such as exosporium, pure enzyme would allow these measurements to be made in the absence of other cellular

components (e.g. other proteins, lipids, and polysaccharides), and therefore provide a view of nanoscale interactions between enzyme and biogenic mineral.

An ultimate goal of molecular biogeochemistry of Mn(II) oxidation research is to define which organisms and enzymes drive this cycle in nature. I believe that reaching this goal will require the continued application of integrated approaches in biogeochemistry, environmental microbiology, and physiology/biochemistry of Mn(II) oxidation by model organisms. However, there are several approaches that could be applied in the near-term that I feel are promising. First, in environments like the GB deep-sea hydrothermal plume where particulate Mn oxide is dominated by single Mn-encrusted cells, it should be possible to physically separate these cells from other cells and particles. I attempted to do this by percoll-gradient density centrifugation of concentrated Mn oxides, however a major problem was clumping of Mn oxides to each other and possibly other bacterial cells that are not involved in Mn oxidation. Therefore the separation should be attempted on unconcentrated seawater, probably with cell-sorting technologies such as flow cytometry. If the physical properties of Mn-encrusted cells are not sufficient to distinguish them from other particles by cell sorting, then fluorescent probes for Mn oxides (e.g. peptides that specifically recognize biogenic Mn oxide) could be developed. Advances in such technologies would not only allow direct identification of natural populations of Mn(II)-oxidizing microbes, it would also allow metagenomic analysis of these populations.

Finally, another major focus of future research on microbial Mn(II) oxidation should be the development of genetic probes for Mn(II) oxidase genes that could be used to interrogate natural samples. The emerging view is that while multicopper oxidases

represent a universal mechanism of bacterial Mn(II) oxidation, Mn(II) oxidizing enzymes from different types of Mn(II) oxidizers are often not similar to each other aside from at conserved Cu-binding regions. A key question is whether Mn(II)-oxidizing multicopper oxidases can be distinguished from other multicopper oxidases (that do not oxidize Mn) based on nucleotide or amino acid sequences. Answering this question requires continued studies of the diversity of Mn(II)-oxidizing bacteria and their molecular mechanisms, as well as more biochemical studies of the specificity of Mn(II) oxidases and other multicopper oxidases. It may be that while universal probes for Mn(II) oxidases are not feasible, group-specific probes (e.g. for *Bacillus*, γ-proteobacteria, α-proteobacteria) can be developed.

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