Direct Identification of a Bacterial Manganese(II) Oxidase, the Multicopper Oxidase MnxG, from Spores of Several Different Marine Bacillus Species

Gregory J. Dick,‡ Justin W. Torpey, Terry J. Beveridge,§ and Bradley M. Tebo

Marine Biology Research Division, Scripps Institution of Oceanography, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0202; Biomolecular Mass Spectrometry Facility, Department of Chemistry and Biochemistry, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0578; and Department of Molecular and Cellular Biology, College of Biological Science, University of Guelph, Guelph, Ontario N1G 2W1, Canada

Received 4 June 2007/Accepted 20 December 2007

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 to directly analyze the Mn(II)-oxidizing enzyme from marine Bacillus spores, identified as an 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 sodium dodecyl sulfate. 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 direct 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 from Mn(III) to Mn(IV), a novel type of reaction for a multicopper oxidase.

Mn(III,IV) oxides are reactive minerals that play an important role in the global cycling of many major (C and S) and trace elements (Fe, Co, Pb, Cu, Cd, and Cr) in nature (52). 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 (32, 52). This microbially mediated Mn(II) oxidation occurs by an enzymatic pathway (13, 42), 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 (8, 17, 30, 51, 52), 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 (4), Leptothrix discophora strain SS-1 (7), Bacillus sp. strain SG-1 (55), and Pedomicrobium sp. strain ACM 3067 (38), suggesting a universal mechanism of bacterial Mn(II) oxidation. MCOs are a family of enzymes that use multiple Cu atoms—classified into three types of Cu sites—as cofactors in coupling the oxidation of substrate to the reduction of O2 to H2O (46). MCO substrates include a variety of organic compounds, as well as metal ions such as Fe2+, 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 to completion have failed (1, 22a, 35; D. B. Edwards and B. M. Tebo, unpublished data), as have efforts to produce active Mn(II) oxidase by expressing MCO genes in heterologous hosts (5, 12). 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 (6, 46), including iron and copper homeostasis (21, 37, 50), siderophore oxidation (16), pigment formation (20), and biopolymerization (47). 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 the present study, we investigated the role of an MCO in Mn(II) oxidation by marine Bacillus spores. Phylogenetically diverse Mn(II)-oxidizing Bacillus species have been isolated from a number of environments in which Mn(II) oxidation is prevalent, such as coastal sediments (14), deep-sea hydrothermal vents (11), and the suboxic zone of the Black Sea (G. J. Dick and B. M. Tebo, unpublished results). These Bacillus species oxidize Mn(II) as metabolically dormant spores (not as

§ Deceased 10 September 2007.
† Supplemental material for this article may be found at http://aem.asm.org.
‡ Present address: Department of Geological Sciences, University of California San Diego, 9500 Gilman Drive, La Jolla, California 92093-0202.
† Corresponding author. Mailing address: Department of Environmental and Biomolecular Systems, OGI School of Science and Engineering, Oregon Health and Sciences University, 20000 NW Walker Rd., Beaverton, OR 97006. Phone: (503) 748-1992. Fax: (503) 748-1464. E-mail: tebo@ebs.ogi.edu.
Growing or vegetative cells) via an unidentified Mn(II)-oxidizing enzyme (42) located in the exosporium, the outermost layer of the spore coat (12). 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 (55). One of these genes, mnxG, encodes a predicted protein with Cu-binding motifs 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 of Mn(III) to Mn(IV) (57). The possibility that MnxG is the direct catalyst raises interesting mechanistic questions because all well-characterized MCOs catalyze one-electron transfer reactions (46), 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 phylogenetic diversity of marine Mn(II)-oxidizing Bacillus spores has been recognized (11, 14). The Mn(II) oxidase of these diverse Mn(II)-oxidizing Bacillus spores can be visualized by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in-gel activity assay, and its size varies widely among species (14). Here we describe cloning and DNA sequence analysis of the mnx gene cluster from two diverse Bacillus strains—PL-12 and MB-7—and comparative sequence analysis with strain SG-1. DNA sequence of the mnx region also facilitated interpretation of tandem mass spectrometric (MS/MS) analysis of the Mn(II) oxidase, leading to the most prominent finding of our research—the first direct identification of a bacterial Mn(II) oxidase.

**MATERIALS AND METHODS**

**Growth, DNA extraction, cloning, and sequencing.** Bacillus cultures were grown in K medium as described previously (53) with shaking (150 rpm) at room temperature. DNA was isolated by phenol-chloroform extraction (18). The PL-12 mnx region was cloned by a combination of 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, fractionation of the HindIII fragments on a 10 to 40% (wt/vol) sucrose density gradient (28), selection of 6- to 8-kb fragments by agarose gel-electrophoresis (SDS-PAGE) in-gel activity assay, and its size varies widely among species (14). Here we describe cloning and DNA sequence analysis of the mnx gene cluster from two diverse Bacillus strains—PL-12 and MB-7—and comparative sequence analysis with strain SG-1. DNA sequence of the mnx region also facilitated interpretation of tandem mass spectrometric (MS/MS) analysis of the Mn(II) oxidase, leading to the most prominent finding of our research—the first direct identification of a bacterial Mn(II) oxidase.

**Transmission electron microscopy (TEM).** Spores were chemically fixed with 2% glutaraldehyde, followed by 1% osmium tetroxide, stained en bloc with 2% uranyl acetate, dehydrated through an ethanol series, and embedded in LR White resin. Thin sections were stained with uranyl acetate and lead citrate before imaging in a Philips EM300 operating under standard conditions at 60 kV.

**SDS-PAGE and in-gel digestion.** SDS-PAGE analysis and the in-gel Mn(II) oxidation assay were done as described previously (14). For strain PL-12, the partially purified Mn oxidase was analyzed, whereas crude exosporium extracts were used for strains MB-7 and SG-1. For strain SG-1, a 2.5% stacking gel was required to allow the Mn(II) oxidase to enter the gel. The darkest portions of the Mn(II) oxidase bands were excised from the gel, washed with 200 μl of H2O, and vortexed for 10 min before removal of the 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 vortex rotated at room temperature for 1 h, and the supernatant was removed. The gel piece was dehydrated with 100 μl of ACN (room temperature, 10 min), the ACN was removed, and 400 μg of ice-cold trypsin (Promega) in 25 mM ammonium bicarbonate–5 mM DTT–50 mM acetonitrile (ACN), vortex mixing for 10 min, and removal of the supernatant. Finally, the gel piece was washed again with water. Samples were then taken through two cycles of mixing with 200 μl of 25 mM ammonium bicarbonate–5 mM DTT–50 mM acetonitrile (ACN), vortex mixing for 10 min, and removal of the supernatant. In another 10 min, the supernatant was removed and saved. A total of 20 μl of 20% ACN–0.1% TFA was added to the sample, which was vortexed again at room temperature for 30 min. The supernatant was removed and combined with the supernatant from the first extraction.

**LC-MS/MS analysis.** Trypsin-digested peptides extracted from SDS-PAGE as described above were analyzed by liquid chromatography (LC)-MS/MS with electrospray ionization. All electrospray ionization experiments were performed by using a QSTAR-XL hybrid mass spectrometer (AB/MDS Sciex) interfaced to a nanoscale reversed-phase high-pressure liquid chromatograph (Famos/Ultimate/switchos; LC Packings) using a 2105BS column (10 cm by 75 μm; Grace-VyDAC) packed with 3-μm C18 beads. The buffer compositions were as follows. Buffer A was composed of 98% H2O, 2% ACN, 0.1% formic acid, and 0.01% TFA; buffer B was composed of 98% ACN, 2% H2O, 0.1% formic acid, and 0.01% TFA. A gradient of 8% buffer B was at a flow rate of 30 μl/min. After a 3-min wash, the Switchos valve was switched, and the peptides were backflushed onto the analytical column and eluted with a 20-min linear gradient from 8% to 100% buffer B at a flow rate of 200 nMl/min. Peptides from keratin contamination and trypsin autolysis were identified and excluded from further analyses. Searches were performed using ProSequest with exclusion lists of previously observed keratin and/or trypsin autolysis peaks.

**Database search and analysis.** Translated mnx gene sequences were used to search MSMS data for mnx peptides by using AnalytIQ 1.1/ProQ 1.1 (49) and BioAnalyt 1.1.5/ProBlast 1.1 (43: Applied Biosystems). To ensure statistically meaningful results, the MS/MS spectra were also tested against a database in which the mnx gene sequences were inserted into SwissProt. Each nonrelated MS/MS spectrum was 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 manually sequenced de novo.

**Nucleotide sequence accession numbers.** Nucleotide sequences of mnx genes were deposited in GenBank with the accession numbers EF158106 and EF158107.
amino acids are identical. Intriguingly, this stretch includes a
region F) (15, 55) is conserved among the three Bacillus
spp., strains PL-12 and MB-7, and SG-1 (Fig. 3a). mnxG is the most highly conserved gene (65 to 70%
predicted amino acid identity between the three strains), whereas other predicted proteins show 30 to 49%
amino acid identity (Table 1). The length of the mnxG gene is nearly identical in all three strains; therefore, the apparent difference in the size of the Mn(II) oxidases (Fig. 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 (46, 55) (labeled A to D in Fig. 3). A fifth putative consensus Cu-binding region near the C terminus of the protein (Fig. 3 region F) (15, 55) is conserved among the three Bacillus spp., a finding consistent with a critical functional role for those amino acids. Another prominent region of conservation occurs at the C terminus of MnxF (Fig. 3a region E), where 12 of 14 amino acids are identical. Intriguingly, this stretch includes a
sequence that resembles an MCO Cu-binding region (Fig. 3b, region E). 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. 3b, 1 to 4). Based on the homology of MCO Cu-binding regions, the extra Cu-binding amino acids found in MnxF and MnxG are predicted to be ligands at all three types of Cu sites present in MCOs (Fig. 3b, regions E, F, and 1 to 4).

Partial purification of the Mn(II) oxidase 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, Triton X-100, CHAPS, Tween 20, NP-40, SDS, etc.) at various concentrations and conditions (NaOH, NaCl, glycerol, DTT, and 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 (10) and Mn(II) oxidases (13, 35). Efforts to remove the SDS or exchange it for another detergent and maintain activity were unsuccessful, thereby limiting purification options.

The Mn(II)-oxidizing activity from PL-12 is resistant to many proteases in solution. Of the proteases tested (pronase, trypsin, Glu-C, Arg-C, Asp-N, and 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 exosome extract with trypsin, Mn(II)-oxidizing activity was unaffected or in some cases even enhanced, whereas nearly all other exosome proteins were digested (Fig. 4). After solubilization and size-exclusion chromatography of the trypsin-digested exosporium, the specific activity increased >50-fold from the solubilized crude exosporium (2 nmol of MnO₂ equivalents h⁻¹ A₇50⁻¹) to the most pure fraction (119 nmol MnO₂ equivalents h⁻¹ A₇50⁻¹) (we report specific activity per A₇50 rather than per mg of 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 blue band corresponding to the Mn(II) oxidase is faint, and several other bands are still present (Fig. 4).

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 nanoscale LC MS with MS/MS analysis. Although no pertinent peptides were recovered from the crude PL-12 exosporium, five distinct MnxG peptides were repeatedly detected from the

![FIG. 1. SDS-PAGE of exosporium extracts from three Bacillus strains, stained with Coomassie blue (left) and an in-gel Mn(II) oxidation activity assay (right).](http://aem.asm.org/)

![FIG. 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. Scale bars, 200 nm.](http://aem.asm.org/)
FIG. 3. (a) Schematic representation of mnx region genes of Bacillus sp. strains SG-1, PL-12, and MB-7. The locations of putative Cu-binding regions are indicated by open rectangles and labeled A to F according to the data in panel b. Additional regions with potential Cu ligands are indicated by numbers (1–4) as in panel b. The locations of peptides detected by MS/MS analysis of the Mn oxide band from the in-gel activity assay are shown with black bars beneath the mnx genes of strains PL-12 and MB-7. (b) Alignment of putative mnx Cu-binding region amino acid sequences to consensus Cu-binding sequence motifs found in all MCOs. The consensus sequences shown (I to IV) 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 P. putida (CumA) and L. discophora (MofA). "AA#" refers to the number of the first amino acid of each block. Note that the order of the MnxG Cu-binding regions (C, D, A, and B) is different than all other MCOs (III, IV, I, and II). Consensus MCO Cu-binding motifs are highlighted in black, and the type of Cu site for each ligand (based on characterized MCOs) is indicated with T1, T2, or T3.
partially purified PL-12 Mn(II) oxidase (see Fig. S1 in the supplemental material and Table 2). Four different MnxG peptides and one MnxF peptide (the C-terminal tryptic peptide) were consistently recovered from the Mn oxide band from the crude exosporium of strain MB-7 (Table 2). The MnxG peptides detected from PL-12 and MB-7 cluster toward the C terminus of the predicted protein sequence (Fig. 3a). No other Bacillus proteins were detected in the Mn oxide band of PL-12 or MB-7. A higher-molecular-mass band (~140 kDa) often present in the in-gel Mn(II) oxidation assay of strain MB-7 (Fig. 1) and occasionally PL-12 (data not shown) did not yield any detectable peptides, and 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. 3a), and there was a high incidence of peptides from trypsin autolysis (trypsin digesting itself) and nonspecific cleavages (having a tryptic cleavage at one end and a nonspecific cleavage at the other). A total of 74 MS/MS spectra obtained from strain PL-12 were de novo sequenced, and 35 spectra were of sufficient data quality for interpretation. Thirty of these peptides were from trypsin, and only five spectra were from MnxG. Two of five MnxG peptides had nonspecific cleavages (LEFVLLLHDGVR and KAPRPPLGIV), as did one of the MB-7 peptides (Table 2).

**DISCUSSION**

MCOs are a large family of enzymes with over 500 putative homologs in all three domains of life. Although all MCOs couple the oxidation of substrate to the reduction of O2 to H2O, their substrates (organics versus metals) and specificity (narrow versus 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 the spores of two Bacillus spp. identified nine different peptides unique to the MCO MnxG and one peptide unique to the small hydrophobic protein MnxF. These peptides were repeatedly detected through multiple analyses, and 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 formation and therefore is the direct catalyst of Mn(II) oxidation in the exosporium. 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 MCO MnxG and one peptide unique to the small hydrophobic protein MnxF. These peptides were repeatedly detected through multiple analyses, and 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 formation and therefore is the direct catalyst of Mn(II) oxidation in the exosporium. 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. Although 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 (34). 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.

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

<table>
<thead>
<tr>
<th>Predicted protein</th>
<th>Amino acid sequence identity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SG-1/PL-12</td>
</tr>
<tr>
<td>MnxA</td>
<td>46.6</td>
</tr>
<tr>
<td>MnxB</td>
<td>34.0</td>
</tr>
<tr>
<td>MnxC</td>
<td>**</td>
</tr>
<tr>
<td>MnxD</td>
<td>36.1</td>
</tr>
<tr>
<td>MnxE</td>
<td>49.1</td>
</tr>
<tr>
<td>MnxF</td>
<td>43.1</td>
</tr>
<tr>
<td>MnxG</td>
<td>68.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Present only in strains PL-12 and SG-1; ** present only in strain SG-1.

**TABLE 2. Peptides identified by MS/MS analysis of the Mn(II) oxidase**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Protein</th>
<th>Peptide sequence</th>
<th>Amino acid position</th>
</tr>
</thead>
<tbody>
<tr>
<td>PL-12</td>
<td>MnxG</td>
<td>AITGENVILR</td>
<td>1011–1020</td>
</tr>
<tr>
<td></td>
<td>MnxG</td>
<td>SGFTFGAFVASESR</td>
<td>986–998</td>
</tr>
<tr>
<td></td>
<td>MnxG</td>
<td>EFLVLLLHDGVR</td>
<td>1024–1034</td>
</tr>
<tr>
<td></td>
<td>MnxG</td>
<td>MPHILDGDAFQLVTR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>508–522</td>
</tr>
<tr>
<td></td>
<td>MnxG</td>
<td>APRPPLGIV</td>
<td>402–410</td>
</tr>
<tr>
<td>MB-7</td>
<td>MnxG</td>
<td>EFLVLMYDGR</td>
<td>1024–1034</td>
</tr>
<tr>
<td></td>
<td>MnxF</td>
<td>ANQGDVIEINLTSR</td>
<td>884–897</td>
</tr>
<tr>
<td></td>
<td>MnxF</td>
<td>GSLAQLLDYDVK</td>
<td>927–939</td>
</tr>
<tr>
<td></td>
<td>MnxF</td>
<td>SLGTFGAFIAEPK</td>
<td>986–998</td>
</tr>
<tr>
<td></td>
<td>MnxF</td>
<td>IPELDDDF</td>
<td>95–102</td>
</tr>
</tbody>
</table>

<sup>a</sup> Only the five C-terminal residues show unambiguous MS/MS sequence for this peptide.
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 nonspecific cleavages and trypsin autolysis peaks in the MS/MS spectra are also consistent with the low concentration of MnxG protein. These observations, including the absence of a clear Coomassie blue stainable band, suggest that there was at most 10 to 20 ng (ca. 100 to 200 fmol) of MnxG protein present in the gel band. Another possibility is that MnxG is resistant to trypsin digestion; the relatively high occurrence of nonspecific cleavages that we observed suggests that peptides were produced with low efficiency. Although there are many tryptic cleavage sites (arginine or lysine) in MnxG, trypsin 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 of other Bacillus spores (23).

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 apparent molecular mass, which limits its entry into the SDS-PAGE resolving gel. The larger apparent 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). Other intrinsic properties of MnxG that might influence the migration by SDS-PAGE, such as isoelectric point and hydrophobicity, are very similar among the three strains (as calculated by amino acid sequence [data not shown]) and thus not expected to be determining factors. The SG-1 Mn(II) oxidase may occur as a high-molecular-mass complex because of the association of additional subunits and/or proteins or because of differences in the overall structure of the exosporium, the outermost layer of the spore coat in which the Mn(II) oxidase is located (12). TEM shows that these three strains 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. 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, and yet, as the site of Mn oxide deposition and 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) (57), 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 site. 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 O2 to 2H2O (46). 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 MCO 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) (36). After oxidation, Fe(III) is translocated to a “holding site,” where it is then donated to transferrin (hCp) or Ftr1p (Fet3p) (3, 26, 31, 56). 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 (50) and, of the three tested to date (P. aeruginosa MCO, CueO, and Fet3p), none are able to oxidize Mn(II) (21, 24). In contrast, laccases lack a substrate-binding pocket, exhibiting broad substrate specificity that correlates with the substrate’s oxidation potential (46). Fungal laccases are capable of oxidizing Mn(II) to Mn(III) (44), in some cases producing Mn(IV) oxide (29), and yet it is currently unclear whether this Mn(IV) is produced enzymatically or by the disproportionation of Mn(III). Thus, MnxG is unique among MCOs in its ability to catalyze the oxidation of Mn(II) to Mn(IV). 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 (2). However, the enzymatic mechanism of this multielectron transfer is not understood.

The MnxF/G proteins contain a unique set of Cu-binding regions not present in any characterized MCOs but most similar to hCp (Fig. 3). Homologues of histidines within these regions are required for activity in other MCOs (50), and structural data have confirmed their role in binding Cu (39, 50). Based on sequence homology, the extra Cu-binding ligands of MnxF/G (Fig. 3, regions E, F, and 1 to 4) are predicted to coordinate Cu at all three types of Cu sites. A tally of these putative extra Cu ligands yields 8 type 1, 6 type 2, and 10 type 3 ligands, whereas the typical MCO (with four Cu atoms) has 4 type 1, 2 type 2, and 6 type 3 ligands. It is unclear whether all of these predicted ligands actually bind Cu. Additional Cu ligands that have not yet been detected could lie elsewhere in the MnxG amino acid sequence. Still, an intriguing possibility is that 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 atoms; it has two extra Cu atoms that are both type 1 Cu, although it is redox active, is unclear. Other MCOs with extra Cu atoms include CueO (E. coli), where the extra Cu is involved in regulation (40), and phenoxazinone synthase, where the extra Cu is thought to stabilize quaternary structure (45). These cases are distinguished from MnxG because the Cu atoms are coordinated by histidines and...
metionines 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 im-
portant environmental consequences. MnxG’s unusual com-
plement of putative Cu ligands and the two-electron reaction
that it catalyzes suggest that it is a novel MCO that may
implement of putative Cu ligands and the two-electron reaction
important environmental consequences. MnxG’s unusual com-
plement of putative Cu ligands and the two-electron reaction
important environmental consequences. MnxG’s unusual com-
plement of putative Cu ligands and the two-electron reaction

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
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 thus far efforts to express MnxG in E. coli
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.

ACKNOWLEDGMENTS

We thank members of the Tebo laboratory for helpful critical review of
the manuscript, Dianne Moyle of T. J. Beveridge’s laboratory, and
Chris Francis for the preparation of spores for electron microscopy.

This research was supported by grants to B.M.T. from UCSD’s
Superfund Basic Research Program grant (NIEHS ES10337), NSF
Ocean Sciences (OCE-0352081/0635493), and the NSF CREAMS
program (NSF CHE-0809208). G.J.D. was supported in part by a NSF
graduate research fellowship. T.J.B.’s research is supported by a US-
DOE-NABIR grant. The electron microscopy was performed in the
NSERC Guelph Regional Integrated Imaging Facility, which is par-
tially supported through an NSERC Major Facilities Access grant to
T.J.B.

REFERENCES

Mn(II)-oxidizing activity and isolation of Mn(II)-oxidizing protein from
Lepto-
mechanism for the formation of the phenoxazinone chromophore of actino-
1999. Homology modeling of the multicopper oxidase Fet3p gives new insights
Bayse, and E. W. de Vrind-de Jong. 1999. "cmeC, a gene encoding a multi-
copper oxidase, is involved in Mn(II)-oxidation in Pseudomonas putida GB-1.


40. Reference deleted.


