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Localization of Mn(II)-oxidizing activity and the putative multicopper oxidase, MnxG, to the exosporium of the marine *Bacillus* sp. strain SG-1

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

Keywords Mn(II) oxidation · *Bacillus* · spores · Exosporium · MnxG · multicopper oxidase

Introduction

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

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

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

Materials and methods

Bacterial strains, media, and culture conditions

The bacterial strains used in this study were the *Bacillus* sp. strain SG-1 and various Tn917 manganese oxidation mutants generated by van Waasbergen et al. (1993). The sporulation medium for SG-1 is a modified K medium (Rosson and Neelson 1982) containing 2.0 mg of peptone (Difco) per ml and 0.5 mg of yeast extract (Difco) per ml in 75% sterile seawater with both 20 mM HEPES (pH 7.6) and 100 μ M MnCl₂ added after autoclaving.

Isolation of Mn(II)-oxidizing layer

Spores were purified by the method of Rosson and Neelson (1982). The outermost layer was isolated by passing purified spores through a French pressure cell six times at ~138 MPa. Stripped spores were then removed by centrifugation at 14,000 \times g, and the supernatant, containing most of the activity, was recovered. Supernatant was assayed for Mn(II)-oxidizing activity by incubation in 10 mM HEPES (pH 7.6) containing 100 μ M MnCl₂ and observing the formation of brown Mn oxides.

Electron microscopy of spores

Purified spores (French press-treated and untreated) were incubated in 10 mM HEPES-buffered distilled water with 100 μ M MnCl₂ for 3 h at room temperature and fixed in glutaraldehyde/cacodylate buffer at 4 °C overnight. Fixed spores were then washed twice with cacodylate buffer, postfixed in 1% OsO₄, embedded in agar, dehydrated through a graded ethanol series (30%, 50%, 60%, 70%, 80%, 90%, 3 \times 95%, 3 \times 100%), and embedded in Spurr's resin. Blocks were sectioned, stained with 2% uranyl acetate and Reynold's lead citrate, and viewed with a JEOL1200-EXII transmission electron microscope at 60 kV accelerating voltage.

Chemical composition analysis

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

alyzed by the UC San Diego Glycotechnology Core Research Lab. Samples were hydrolyzed in 2 M trifluoroacetic acid at 120 °C for 3 h and analyzed by HPLC. The response was normalized to standards of glucose, galactosamine, glucosamine, xylose, rhamnose, and mannose subjected to the same hydrolysis treatment. Lipids were extracted by the standard method of Bligh and Dyer (1959) in a chloroform/methanol/water system. Total lipid content was determined by charring in H₂SO₄ at 200 °C followed by spectrophotometric quantification against tripalmitin standards (Marsh and Weinstein 1966).

Prior to fatty acid methyl ester (FAME) derivatization, samples (outer layer, spore coat, and vegetative cell pellets) were frozen at -80 °C overnight and lyophilized. FAME analysis of this material was performed as described previously (Allen et al. 1999). FAMES were prepared by reacting 2.5–10 mg (dry weight) of lyophilized sample with 5% H₂SO₄ in anhydrous methanol at 90 °C for 90 min in 1.5-ml sample vials with Teflon-lined caps. FAMES were extracted twice with hexane and nonesterified fatty acids were saponified with 10% NaCl. Hexane layers were removed and evaporated under a gentle stream of N₂ prior to analysis. The FAME preparations were analyzed on a Hewlett-Packard model 5890 gas chromatograph equipped with an Econo-Cap EC-Wax capillary column (30 m \times 0.25 mm ID \times 0.25 μ m) connected to an HP model 5988A mass spectrometer. Compounds were identified by comparison of their retention times with those of known standards and sample mass spectra data were compared to the mass spectra of known standards. Fatty acids are denoted as number of carbon atoms: number double bonds. Branched-chain fatty acids were not resolved to iso- and anteiso- species and have been combined as branched-chain fatty acids only.

SDS-PAGE analysis

Supernatant containing the outermost layer was mixed with 2 \times Laemmli buffer (Laemmli 1970) and proteins were separated by SDS-PAGE in standard 6% or gradient (2.5–15%) polyacrylamide gels, followed by staining with Coomassie blue. To assay for in-gel Mn(II)-oxidation activity, gels were first incubated in 0.5% Triton-X-100/10% glycerol for 30 min to remove SDS, and then incubated in 10 mM HEPES buffer (pH 7.6) containing 100 μ M MnCl₂ (Francis and Tebo 2002). Mn(II) oxidation was visualized by the formation of brown Mn oxide bands in gels after several hours of incubation.

Overexpression of MnxG and generation of polyclonal antibodies

The *mnxG* gene was amplified with PCR primers containing engineered *Kpn*I sites, restriction digested, and cloned into the expression vector pTRXFus (Invitrogen), creating an in-frame fusion with the *E. coli* thioredoxin gene. The fusion protein expressed from tryptophan-induced cells, which constituted most of the insoluble fraction of the *E. coli* cell lysates, was electrophoresed in 6% polyacrylamide gels, and bands of the MnxG fusion protein were excised from KCl-stained gels and used for the production of polyclonal antisera in chickens (Cocalico Biologicals). Antibodies were preadsorbed with an acetone powder of *E. coli* (pTRXFus) in which thioredoxin had been expressed, followed by affinity purification using expressed MnxG immobilized on nitrocellulose blots (Harlow and Lane 1988).

Western blot analysis

Following SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose for Western blot analysis. Prestained molecular weight markers (Sigma) were used as standards. The primary antibody used for probing Western blots was purified anti-MnxG chicken polyclonal antiserum (1:1,000), and the secondary antibody was a rabbit anti-chicken-IgY horseradish peroxidase conjugate (Jackson Immunochemicals). Blots were developed using enhanced chemiluminescence (DuPont-NEN).

Results and discussion

Localization of Mn(II)-oxidizing activity

The Mn(II)-oxidizing activity of SG-1 spores was reduced by approximately 50% after several passages through a French press at ~138 MPa, centrifugation, and removal of the supernatant. A significant amount of the activity lost from the spores was recovered in the supernatant (Fig. 1), indicating that some component of the spores that was responsible for the activity was being removed or stripped off. French press treatment is recognized as an effective method for removing the exosporium, an outermost spore layer found in certain *Bacillus* and *Clostridium* species, without disrupting the integrity of the remaining spore layers (e.g., spore coat) (Charlton et al. 1999; Matz et al. 1970). Previous studies of SG-1 indicated that Mn(II) oxidation is catalyzed by a protein component on the spore surface (de Vrind et al. 1986); however, no further localization of this activity to the spore coat or an exosporium was made. In this study, the removal of Mn(II)-oxidizing activity from SG-1 spores by mechanical shear suggested



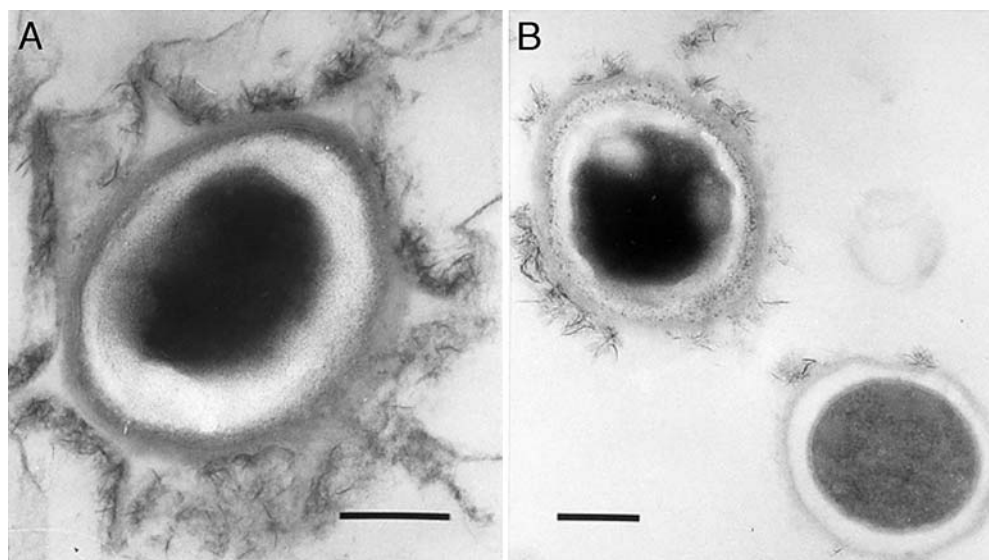
Fig. 1 Recovery of Mn(II)-oxidizing activity in the supernatant following French press-treatment of wild-type spores (*left*) but not in spores of a non-oxidizing mutant (*right*). Supernatants were incubated in HEPES buffer (pH 7.6) containing 100 μ M MnCl₂, resulting in the formation of brown colloidal Mn oxides in the wild-type sample

that an exosporium protein might be responsible for this activity.

The effect of French press treatment on the ultrastructure of SG-1 spores was visualized by TEM (Fig. 2). SG-1 spores are ultrastructurally similar in appearance to *Bacillus subtilis* spores but with an additional ridged layer outside the electron-dense spore coats. Incubation of the spores in a Mn(II) buffer prior to fixation resulted in the formation of Mn oxides, visible as black needle-like or platy precipitates, on the spore surface (Fig. 2A). The pronounced ridges, clearly associated with the Mn oxides in the untreated spores, were almost completely removed in the French-press-treated spores (Fig. 2B). However, some of the outermost layer was still present after French press treatment, as evidenced by the small patches of Mn oxides still associated with the spore surface. These patches may represent attachment sites or anchors for this outer layer, which are not easily removed by mechanical shear. It should also be noted that not all spores passed through the French press are completely stripped of the outermost layer. In fact, Matz et al. (1970) found that, after passage of *Bacillus cereus* spores through a pressure cell at ~221 MPa, only 35% were totally stripped, 50% were partially stripped, and the remainder were left intact. The fact that the outermost layer is only partially removed by the French press (Fig. 2B) explains why some Mn(II)-oxidizing activity is still associated with spores following this treatment.

The spore coat is a highly cross-linked proteinaceous structure that gives the spore resistance to chemical attack and mechanical disruption (Driks 1999; Warth 1978). The exosporium is an additional spore layer found only in certain *Bacillus* and *Clostridium* species, defined as a loose-fitting, membranous layer composed of protein, lipid, and carbohydrate (Matz et al. 1970; Tipper and Gauthier 1972). Chemical analysis of the outermost layer of SG-1 spores demonstrated that it was composed of protein, carbohydrate, and lipid (Table 1). Although it is evident from TEM that the spore coat fraction is contaminated with the outermost layer, these two layers had some distinguishing chemical properties. There were a number of minor differences in the relative amino acid compositions of the outermost layer and the spore coat as well as a few more notable differences (Table 2). Based on molar ratios, the outermost layer appeared to have higher amounts of several amino acids, including leucine, methionine, alanine, isoleucine, phenylalanine, and glutam-x (glutamine or glutamic acid). In addition, there was a higher concentration of tyrosine in the spore coat, which is consistent with previous studies of *B. subtilis* indicating the presence of tyrosine-rich spore coat proteins (Pandey and Aronson 1979) as well as dityrosine cross-links in this layer (Driks 1999). The primary monosaccharides present in both layers were very similar in type and abundance (Table 1). The sugars found (in order of decreasing abundance) were glucosamine, galactosamine, glucose, mannose, galactose, and xylose. The absence of muramic acid in both layers suggests lack of contamination with cortical glycopeptide. The crude lipid content was also similar in both layers. The similar amounts of lipid and carbohydrate in both layers may be

Fig. 2A, B Transmission electron micrograph demonstrating the effect of French press-treatment on the ultrastructure of SG-1 spores. The ridged outermost layer surrounding the untreated spores (A) is partially removed by the French press (B). Remnants of the outer layer can be visualized by the patches of black Mn oxide precipitates still associated with the stripped spores. Bars 0.25 μm



due in part to incomplete removal of the outermost layer by the French press.

There is some uncertainty regarding what may constitute the remainder of the dry weight of the outermost layer. Previous studies of exosporia have indicated the presence of minor components such as ash, nucleic acids, teichoic acid, ammonia, and phosphorus (Matz et al. 1970). Evidence for the presence of phosphorus in the outermost layer of SG-1 spores comes from previous studies which predicted that phosphate and carboxylate groups were primarily responsible for the surface charge and copper adsorption capacity of the spore surface (He and Tebo 1998). Considering the high metal adsorption capacity of the SG-1 outermost spore layer, it is also possible that residual Mn and other inorganic constituents still bound to this layer may have contributed to the overall dry weight. Although the spore coat fraction also likely had some outer layer still associated with it, this fraction was subjected to a number of additional washes during the purification protocol that may have removed any remaining inorganic compounds. FAME analysis revealed distinct differences in the fatty acid composition of the two layers (Table 3).

Table 1 Gross chemical composition of isolated outermost layer and spore coat

Component analyzed	Outermost layer (% dry weight)	Spore coat (% dry weight)
Protein (18 amino acids)	52.48 \pm 2.35	85.51 \pm 1.58
Polysaccharide	4.85 \pm 0.37	4.92 \pm 1.19
Glucosamine	2.98 \pm 0.27	2.88 \pm 0.54
Galactosamine	1.64 \pm 0.08	1.67 \pm 0.39
Glucose	0.13 \pm 0.01	0.22 \pm 0.16
Mannose	0.04 \pm <0.01	0.05 \pm 0.03
Galactose	0.04 \pm 0.01	0.05 \pm 0.04
Xylose	0.02 \pm <0.01	0.05 \pm 0.03
Lipid	6.30 \pm 0.14	7.58 \pm 0.92
Total	63.63 \pm 2.86	98.01 \pm 3.69

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

The gross chemical composition of the *B. cereus* exosporium (Matz et al. 1970) is 52% protein, 20% polysaccharide, 18% lipid, and 4% ash. Compared to that layer, the outermost layer of SG-1 spores contains an almost identical amount of total protein (52.48%) as well as fairly comparable molar ratios of amino acids. These include essentially identical ratios for phenylalanine and leucine, both of which are higher in concentration within the outermost layer than in the spore coat of SG-1 spores. Despite the similarities between the protein fractions of the SG-1 outermost layer and the *B. cereus* exosporium, considerably lower amounts of polysaccharide (4.85%) and lipid (6.3%) were detected in the SG-1 outer layer relative to *B. cereus*. Of the sugars found in the *B. cereus* exosporium (glucose, rhamnose, glucosamine, and ribose), only glucose and glucosamine were also present in the outer layer of SG-1, which also contained significant amounts of galactosamine and smaller amounts of mannose, galactose, and xylose. Comparisons are made with *B. cereus* because this is the only organism for which the chemical composition of the exosporium has been determined. It is possible that the differences in the outermost spore layers of SG-1 and *B. cereus* are due partly to the fact that these two *Bacillus* species are not that closely related phylogenetically and thus might be expected to have notable physiological differences, including exosporium composition. Nevertheless, based on visualization of the outermost layer in SG-1 (Fig. 2), combined with the chemical composition data (Tables 1–3), we conclude that the outermost layer of SG-1 is an exosporium.

Although no definite function has been attributed to exosporia, a number of possible functions have been pro-

Table 2 Amino acid analysis of outermost layer and spore coat

Constituent	Outermost layer		Spore coat	
	Dry weight (%)	Molar ratio ^a	Dry weight (%)	Molar ratio ^a
Alanine	3.48±0.17	0.92±<0.01	4.89±0.165	0.71±0.01
Arginine	3.70±0.15	0.44±<0.01	5.47±0.11	0.36±<0.01
Aspar-x	5.58±0.26	0.91±<0.01	11.85±0.21	1.08±<0.01
Glutam-x	8.39±0.35	1.23±0.01	9.17±0.17	0.74±<0.01
Glycine	3.04±0.16	1.00	5.48±0.08	1.00
Histidine	1.07±0.02	0.15±<0.01	2.38±0.05	0.18±<0.01
Isoleucine	2.34±0.07	0.39±<0.01	3.20±0.07	0.29±<0.01
Leucine	3.94±0.15	0.65±<0.01	5.05±0.09	0.46±<0.01
Lysine	3.31±0.14	0.49±<0.01	6.33±0.10	0.51±<0.01
Methionine	1.42±0.06	0.20±<0.01	1.75±0.02	0.14±<0.01
Phenylalanine	4.67±0.24	0.60±<0.01	6.58±0.06	0.47±<0.01
Proline	1.94±0.14	0.38±<0.01	4.06±0.07	0.43±<0.01
Serine	2.25±0.11	0.49±<0.01	3.67±0.06	0.44±<0.01
Threonine	2.06±0.09	0.38±<0.01	3.44±0.06	0.35±<0.01
Tyrosine	2.64±0.15	0.30±<0.01	7.06±0.17	0.45±<0.01
Valine	2.64±0.08	0.50±0.01	5.13±0.10	0.54±<0.01

^aGlycine=1.0**Table 3** Fatty acid analysis of outermost layer and spore coat

Fatty acid	Vegetative cells (% of total fatty acids)	Outermost layer (% of total fatty acids)	Spore coat (% of total fatty acids)
br14:0	3.79± 0.52	3.74±0.06	17.52±0.41
14:0	3.05±0.10	0.40±<0.01	Trace
14:1	0.74±<0.01	Trace	Trace
br15:0	21.75±1.06	35.00±0.23	29.90±1.53
15:0	43.80±0.58	21.10±0.18	14.31 ±0.37
br16:0	0.88±0.04	1.71±0.27	7.95±0.21
br16:1	6.49±0.44	7.76±0.17	Trace
16:0	0.61±0.01	0.95±0.08	10.45±0.18
16:1	2.57±0.10	2.03±0.20	Trace
br17:0	Trace	1.44±0.03	Trace
17:0	3.31±0.27	14.05±0.64	12.22±1.39
17:1	12.97±0.58	11.73±0.62	7.48±1.60
Total unsaturated	16.28±0.68	13.76±0.01	7.48±1.60
Total branched	32.91±2.06	49.65±0.01	55.37±0.76
Total saturated	50.77±0.96	36.50±0.01	36.98±1.94

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

could potentially facilitate attachment/adhesion to surfaces (Charlton et al. 1999). In fact, surfaces were found to enhance the ability of SG-1 to sporulate and oxidize Mn(II) (Nealson and Ford 1980; Kepkay and Nealson 1982). Overall, it seems somewhat unlikely that the exosporium has an indispensable function since it is not present in spores of all *Bacillus* species. However, the exosporium may have species-specific functions which somehow relate to the ecological niche(s) of a given organism.

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

The functions of these proteins, or how tightly they are associated with the exosporium of *B. cereus*, is currently unknown.

Localization of MnxG

In previous genetic studies of the marine *Bacillus* sp. strain SG-1, transposon mutants were generated that produced spores incapable of Mn(II) oxidation (van Waasbergen et al. 1993). Transmission electron microscopic analysis of these mutants revealed subtle changes in the exosporium (e.g., more loosely attached, peeling/sloughing off), suggesting that some component(s) of this layer had been disrupted (van Waasbergen et al. 1996). However, none of the mutant spores examined completely lacked the exosporium, and no significant differences were found between mutant and wild-type spores in terms of their germination properties or resistance to lysozyme, chloroform, ethanol, and heat (van Waasbergen et al. 1996). Genetic analysis of these non-oxidizing mutants led to the isolation and sequence analysis of a cluster of seven genes, the *mnx* genes, which appeared to be essential for Mn(II) oxidation (van Waasbergen et al. 1996). The most downstream gene in the *mnx* cluster encodes the putative Mn(II) oxidase, MnxG. Regions of this gene product share significant similarity with multicopper oxidases, a diverse group of proteins that use multiple copper ions to oxidize a variety of substrates, the majority of which are organic compounds (e.g., ascorbate, diphenolics, syringaldazine) but also Fe(II) (Solomon et al. 1996). Particularly conserved regions include those that are involved in copper binding. The involvement of a multicopper oxidase in Mn(II) oxidation by SG-1 is supported by the fact that small amounts of copper (1 μ M) enhance the rate of Mn(II) oxidation by SG-1 spores (van Waasbergen et al. 1996). In addition, azide, a potent metalloenzyme inhibitor which bridges the type 2 and type 3 copper atoms of multicopper oxidases (da Silva and Williams 1991), inhibits Mn(II) oxidation by SG-1 spores (Rosson and Nealon 1982).

To further establish the role of MnxG in Mn(II) oxidation by SG-1 spores, we employed a biochemical and immunological approach. The *mnxG* gene was cloned into the pTRXFus (Invitrogen) expression vector, allowing for overexpression of a thioredoxin-fusion protein in *E. coli*. SDS-PAGE analysis of crude lysates revealed a protein of the appropriate size (138+16.5 kDa) in the insoluble fraction (data not shown). However, like the two other bacterial multicopper oxidases involved in Mn(II) oxidation (Brouwers et al. 2000), CumA and MofA, from *Pseudomonas putida* GB-1 and *Leptothrix discophora* SS-1, respectively, no activity could be recovered from the heterologously expressed protein. This may be due to improper refolding of the expressed proteins, the absence of essential cofactors, or the requirement of additional proteins for activity. That the Mn(II)-oxidizing activity of SG-1 exosporium extracts could only be recovered at the stacking/resolving gel interface of low percentage SDS-PAGE gels (Francis and Tebo 2002) suggests the re-

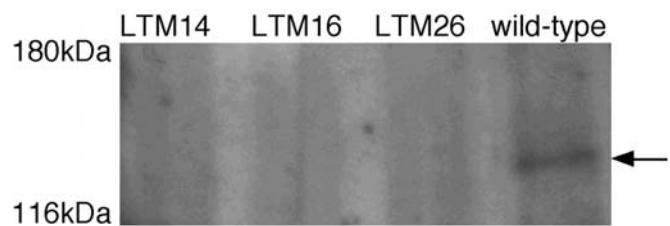


Fig. 3 Western blot of outermost layer extracts from spores of wild-type SG-1 and non-oxidizing mutants (LTM14, LTM16, LTM26) probed with antiserum generated to expressed-MnxG. A band of the expected size (~138 kDa) is present in wild-type extracts but absent in those of the mutants. LTM14 has a transposon insertion in the upstream gene, *mnxB*, while LTM16 and LTM26 have insertions near the beginning and end of *mnxG*, respectively

quirement of a high-molecular-weight multi-protein complex for activity.

Anti-MnxG antibodies, generated using expressed protein as the immunogen, were used in Western blot analysis of the exosporium extracts (Fig. 3). A protein of the expected size (~138 kDa) was detected in wild-type spores, indicating that it was indeed localized to the spore surface layer where the activity was localized. The fact that mutants with transposon insertions in *mnxA-F* exhibit the same non-oxidizing phenotype as those with insertions in *mnxG* suggested that the upstream insertions might simply be exerting a polar effect on *mnxG*. To investigate this further, the exosporium of various *mnx* mutants were also screened for the presence of MnxG via Western analysis (Fig. 3), and MnxG was shown to be absent in each case, suggesting that it may be the only Mnx protein directly involved in Mn(II) oxidation. However, preliminary results from our laboratory suggest that other *mnx* gene products (e.g., MnxC) of unknown function may also be localized to the exosporium (Francis and Tebo, unpublished results). The absence of MnxG (or a smaller truncated product) in spores of even the most downstream insertion mutant (LTM26), with an insertion near the 3' end of *mnxG*, suggests that the full-length polypeptide may be required for proper folding, localization, and activity. In addition, there is also a putative copper-binding site (HTFHLHGH) near the C-terminus of MnxG that may be essential.

The localization of Mn(II)-oxidizing activity and the putative multicopper oxidase, MnxG, to the exosporium of SG-1 spores led us to test spores of several other Mn(II)-oxidizing *Bacillus* isolates for the presence of such a layer. In every case, a significant amount of the activity was recovered in the supernatant following French press treatment, suggesting that the presence of an exosporium may be a common feature of Mn(II)-oxidizing spores. The role of the Mn(II)-oxidizing activity in the exosporium, however, is unknown.

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