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Identification and Characterization of a Gene Cluster Involved in Manganese Oxidation by Spores of the Marine *Bacillus* sp. Strain SG-1

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The marine *Bacillus* sp. strain SG-1 forms spores that oxidize manganese(II) as a result of the activities of uncharacterized components of its spore coat. Nucleotide sequence analysis of chromosomal loci previously identified through insertion mutagenesis as being involved in manganese oxidation identified seven possible genes (designated *mnxA* to *mnxG*) in what appears to be an operon. A potential recognition site for the sporulation, mother-cell-specific, RNA polymerase sigma factor, σ^K, was located just upstream of the cluster, and correspondingly, measurement of β-galactosidase activity from a Tn*917-lacZ* insertion in *mnxD* showed expression at mid-sporulation to late sporulation (approximately stage IV to V of sporulation). Spores of nonoxidizing mutants appeared unaffected with respect to their temperature and chemical resistance properties and germination characteristics. However, transmission electron microscopy revealed alterations in the outermost spore coat. This suggests that products of these genes may be involved in the deposition of the spore coat structure and/or are spore coat proteins themselves. Regions of the deduced protein product of *mnxG* showed amino acid sequence similarity to the family of multicopper oxidases, a diverse group of proteins that use multiple copper ions to oxidize a variety of substrates. Similar regions included those that are involved in binding of copper, and the addition of copper at a low concentration was found to enhance manganese oxidation by the spores. This suggests that the product of this gene may function like a copper oxidase and that it may be directly responsible for the oxidation of manganese by the spores.

The marine *Bacillus* sp. strain SG-1 forms spores that catalyze the oxidation of soluble manganese(II) and thus the deposition of characteristic brownish-black manganese oxides on the spore surface (17, 25, 31, 38, 45, 58). In aerobic environments around neutral pH, the oxidation of manganese is thermodynamically favorable but kinetically slow. It is known that a wide variety of bacteria can facilitate the oxidation of manganese either passively, via an increase in pH or oxygen production, or actively, via the production of proteins or polysaccharides that directly catalyze manganese oxidation (11, 46, 47). The details of the mechanisms whereby most bacteria, including SG-1, are able to catalyze the oxidation of manganese remain largely unknown. Since a large proportion of environmental isolates, including other bacterial spores, have the ability to oxidize manganese (23, 34, 44, 46) and their activities have been shown to have a tremendous impact on the geochemical cycling of manganese (19, 47), it is important to understand how bacteria mediate this process. Also, manganese-oxidizing organisms, particularly manganese-oxidizing spores, may have useful applications for a variety of environmental and industrial metal removal processes (24, 69).

Manganese oxidation by SG-1 spores is heat labile and poisoned by metalloprotein inhibitors (58). Transmission electron microscopy of the spores has shown that the metal precipitates on the outermost, ridged spore coat (68), and manganese oxidation activity has been localized to the spore coat fraction of disrupted spores (17). In addition, when protein extracts of spore coats are run in sodium dodecyl sulfate (SDS)-polyacrylamide gels and presented with manganese(II), a high-molecular-weight manganese-oxidizing band occasionally appears (70). The evidence described above suggests that the oxidizing factor is a component of the spore coat, presumably a spore coat protein.

The bacterial spore coat is believed to be a highly cross-linked structure that encases the spore and gives it its resistance to chemical attack and mechanical disruption (54, 73). The coat is also believed to play a role in the response of the spore to various germinants (42). Eleven genes coding for some of the 20 or so known spore coat proteins in *Bacillus subtilis* have been cloned and sequenced (designated *cot* genes) (4, 12, 18, 26, 61, 74, 76). Most are monocistronic and scattered throughout the chromosome (65), but recently, five proteins of the insoluble fraction of the *B. subtilis* spore coat were found in a cluster (74). Sequence analysis shows that none of the protein sequences shows any similarity to any other known sequences (65), and there is no known sequence similarity among different spore coat proteins suggestive of a spore coat protein motif. The proteins range in size from 5 to 65 kDa (65) and tend to be rich in hydrophobic residues (53). The spore coat genes characterized thus far are under the control of either the σ^K or σ^E subunits of RNA polymerase, the sigma factors controlling gene expression in the mother cell compartment during sporulation (26, 65, 78). Most of the spore coat mutants do not exhibit any loss in resistance or germination characteristics of their spores or show any other altered phenotype except for *cotA* and *cotE* mutants. *cotA* mutants lack a brown pigment

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TABLE 1. Bacterial strains and plasmids used in this study

Strains (or plasmids)	Description	Source or reference
Bacillus sp. strain SG-1		
Wild type		Laboratory collection (45)
LTM1 to LTM27	Tn*917* manganese oxidation (Mnx[a]) mutants	71
SG-1W and WBT	Spontaneous manganese oxidation mutants picked up in routine culturing	Laboratory collection (58)
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lac*^{q}*ZΔM15* Tn*10* (Tc^r)]	Stratagene (8)
Plasmids		
pBR322	Ap^r; *E. coli* cloning vector	5
pSE380	Ap^r; *E. coli* cloning vector with superlinker region	Invitrogen (7)
Recombinant plasmids		
pBRM17N	8.6-kbp *Nde*I, Mnx fragment from SG-1 (previously designated region MnxI) cloned into pBR322	71
pSEM26N	5.1-kbp *Nde*I, Mnx fragment from SG-1 (previously designated region MnxII) cloned into pSE380	This study
pKSP3, pKSP47	4.25-kbp *Hin*dIII-*Cla*I fragment from pBRM17N cloned into pBluescript II KS$^+$ in both orientations	This study
pKSP26BS	1-kbp *Nde*I-*Bam*HI fragment from pSEM26N cloned into pBluescript II KS$^+$	This study
pKSP26BM	1.6-kbp *Bam*HI fragment from pSEM26N cloned into pBluescript II KS$^+$	This study
pLVM7E	Plasmid carrying a 2.0-kbp *Eco*RI chromosomal fragment flanking the transposon insertion from the SG-1 mutant LTM7	71

[a] Mnx is the term applied to the phenotype of manganese oxidation distinct from the process of sporulation.

that is associated with sporulating colonies of *B. subtilis* (18). However, in the context of this report, it should be noted that this coloration is not due to the presence of manganese oxide (71). Spores formed by *cotE* mutants are deficient in a number of outer spore coat proteins, are sensitive to lysozyme, and germinate somewhat more slowly than wild-type spores (76). CotE, besides being a spore coat protein itself, is therefore believed to be involved in assembly of the outer spore coat, perhaps through possible activity as a peroxidase (15, 65).

In previous work, we reported on the development of methods of plasmid transformation and Tn*917* mutagenesis in SG-1 (71). Mutants that still sporulate but whose spores failed to oxidize manganese were isolated, and the majority of insertions generating this phenotype were found to cluster in two regions of the chromosome (designated regions Mnx I and Mnx II). In this report, we extend that study to include nucleotide sequence analysis of the regions and phenotypic characterization of nonoxidizing mutants. Sequencing results show that the two regions Mnx I and II are closely linked, and that within this larger area is located a series of seven open reading frames (ORFs) designated *mnxA* to *mnxG*. Since manganese oxidation is a function of the spore coat, we speculate that some of these genes may encode spore coat proteins or proteins involved in the formation of the spore coat. MnxG, the downstreammost deduced protein in the region, showed amino acid sequence similarity to the family of multicopper oxidases, and copper at low concentrations was found to enhance manganese oxidation by SG-1 spores, suggesting that this protein may be causing manganese oxidation through an activity like that of a copper oxidase.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used during this study are listed in Table 1. SG-1 (wild type) (45) was the source of the chromosomal DNA in the clones used in this report for DNA sequencing. The generation of the nonoxidizing mutants designated LTM1 through LTM27 by use of Tn*917* is described elsewhere (71). Media and culture conditions used for SG-1 were described previously (71). *Escherichia coli* XL1-Blue (8) was used as the host for plasmids used in nucleotide sequence analyses. Unless stated otherwise, all molecular genetic techniques were performed by use of standard protocols (62) or as described previously (71).

The plasmid pBRM17N (71) is an 8.6-kbp *Nde*I fragment from SG-1 (previously designated the Mnx I region [71]) cloned into pBR322 (5); pSEM26N (71) is a 5.1-kbp *Nde*I fragment from SG-1 (previously designated Mnx II [71]) that was blunt ended with the Klenow fragment of *E. coli* DNA polymerase and ligated to *Stu*I-digested pSE380 (7). Other plasmid constructions in preparation for sequencing were as follows. The 4.25-kbp *Hin*dIII-*Cla*I fragment from pBRM17N was blunt ended with the Klenow fragment of *E. coli* DNA polymerase and ligated to *Sma*I-digested pBluescript II KS$^+$ (Stratagene). Plasmids that carry the fragment in both orientations were isolated (pKSP3 and pKSP47). The 1-kbp *Nde*I-*Bam*HI fragment from pSEM26N (the Mnx II region [see Fig. 1]) and the flanking 1.6-kbp *Bam*HI fragment were both cloned separately into pBluescript II KS$^+$ for sequencing (and designated pKSP26BS and pKSP26BM, respectively). The region linking the Mnx I and Mnx II regions is present on a previously cloned *Eco*RI fragment in the plasmid pLVM7E (see Fig. 1) (71).

Nucleotide sequencing. Nested deletions were performed on pKSP3 and pKSP47 with the exonuclease III Erase-a-Base kit (Promega) as directed by the manufacturer. The ligation products of various time points of deletion were used to transform *E. coli* XL1-Blue with calcium chloride-generated competence (62). Individual transformants were picked, their plasmids were isolated, and the plasmids were subjected to restriction digestion analysis to determine the extent of deletion. Clones with overlapping fragments were chosen for sequence analysis. Single-stranded DNA templates were generated by infection with R408 helper phage as recommended by the distributor (Stratagene), and the templates were purified by use of standard protocols (62). The majority of the sequence was obtained from single-stranded pBluescript II DNA templates sequenced by the dideoxy-chain termination method by using [α-^{35}S]thio-dATP and modified T7 DNA polymerase, Sequenase version 2.0 (U.S. Biochemicals Corporation), with sequencing reactions run in denaturing polyacrylamide wedge gradient gels. Portions of the sequence were obtained with an in-house automated DNA sequencer (Applied Biosystems model 373A) with sequencing reactions performed on double-stranded plasmid templates with the AmpliTaq PRISM kit (Applied Biosystems). All sequence data was determined by sequencing on both strands.

Nucleotide and amino acid sequences were analyzed with the facilities available on the VAX/VMS DNASYSTEM, University of California, San Diego (67), and the Wisconsin Genetics Computer Group (16) clusters of molecular biology programs as well as the BLAST sequence comparison program (2) (through the BLAST network service via electronic mail to National Center for Biotechnology Information (NCBI). Amino acid similarities were calculated with the PAM250 matrix (13). Analysis of the statistical significance of alignments of protein sequence was done with either the Protein Information Resource ALIGN program (14) in the case of MnxG versus the copper oxidases or RDF2 (36, 55) for the other putative gene products. Multiple alignment of MnxC and the other proteins in Fig. 3 was made by using CLUSTAL (27, 28). Protein secondary structure prediction was made by use of either the PHDsec method (via electronic mail to European Molecular Biology Laboratory [EMBL]) (59) for MnxG or the Chou-Fasmann (9) and Garnier et al. (22) methods for MnxC.

β-Galactosidase assay. The manganese oxidation mutant LTM2 generated during previous studies (71) carries a Tn*917-lacZ* fusion in the area analyzed in this study. It was the only mutant generated in those studies that has the fusion in the orientation that permits transcription of *lacZ*. LTM2 was grown to sporulation in K medium at 30°C. The culture was monitored for the various stages of sporulation by phase-contrast microscopy at a magnification of ×2,000. Traditionally, the stages of sporulation are defined by the appearance of distinct morphological cell types as visualized by electron microscopy. In *B. subtilis*, the

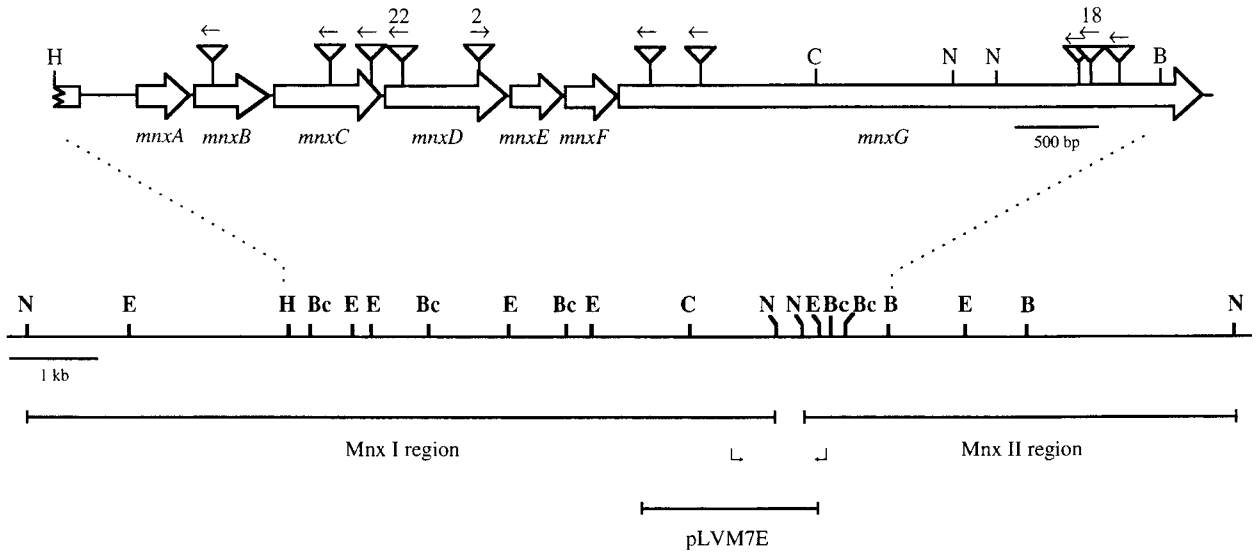

FIG. 1. Restriction map of the region involved in manganese oxidation. The map is shown in relation to the two *Nde*I fragments (Mnx I and II) identified in a previous analysis as being involved in manganese oxidation (71). Mnx I and II were found to be linked by a 250-bp *Nde*I fragment that was discovered by sequencing a clone (pLVM7E [71]) from one of the transposon mutants. The location of the chromosomal DNA carried in pLVM7E is indicated as is the location of primers (bent arrows) used to amplify the intervening region from wild-type SG-1 chromosomal DNA for confirmation of the sequence. Above the map are shown the ORFs identified by nucleotide sequence analysis (arrow boxes), their orientations, and approximate locations of the Tn*917-lacZ* insertions (triangles) that were used to originally identify the loci. The arrows above each transposon insertion indicate the orientations of the transposon-encoded *lacZ* gene, and the numbers of the LTM transposon mutants used in parts of this study are indicated above their corresponding insertions. The only restriction sites shown are those (*Nde*I and *Bcl*I) that help the reader orient the region with previously published maps (71) or those that were used in this study for cloning in preparation for sequencing. Abbreviations: B, *Bam*HI; Bc, *Bcl*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I.

stage number corresponds roughly to the time (t) in hours (x) after sporulation is initiated. In SG-1, the entire sporulation process takes somewhat longer than it does in *B. subtilis*, and so the hours into sporulation do not necessarily correspond to the stage number in this organism. Therefore, to allow comparison of the expression studies done with the two organisms, the latter stages of sporulation in SG-1 were defined by the appearance of cell morphotypes as could be visualized by phase-contrast microscopy (as described in reference 48) as follows. In stage III, the presence of a forespore (a slight darkening) at one end of the cell can be detected. In stages IV to VI, the forespore becomes phase dark and then increasingly phase bright. Samples (1 ml) were collected at hourly intervals, and the cells were harvested by centrifugation. Cell pellets were frozen in a dry ice-ethanol bath and stored at −70°C. The frozen cells were permeabilized with toluene (12) and assayed for β-galactosidase activity with the substrate *o*-nitrophenol-β-D-galactosidase as described by Miller (41).

Electron microscopy. Sporulated colonies or purified free spores of SG-1 and its mutants were prepared for transmission electron microscopy in a manner similar to that described by Tebo (68). Plugs of agar containing colonies from K agar plates (~2 weeks old) or pellets of purified spores (purified by washing with lysozyme and SDS as described by Rosson and Nealson [58]) were fixed in a glutaraldehyde solution containing 3% glutaraldehyde in CAS buffer (0.1 M cacodylate-HCl, 0.4 M NaCl) for 2 h at 4°C. The colonies were trimmed of agar, and the cells or colonies were washed three times for 15 min each time with CAS buffer and then postfixed for 2 h in 1% osmium tetroxide in CAS buffer. The fixed samples were then washed three times with CAS buffer and taken through a graded series of ethanol in distilled water (30, 50, 70, 95, 100, 100, and 100%) followed by two changes of *n*-butyl glycidyl ether (15 min in each solution). The samples were infiltrated with Quetol 651 resin (Electron Microscopy Sciences) and embedded in Durcapan (Fluka) in preparation for sectioning. Blocks were sectioned on an LKB ultramicrotome at 60- to 70-μm thickness and stained with uranyl acetate and Reynold's lead citrate and observed in a Hitachi HT500 electron microscope.

Measurement of Mn(II) binding and oxidation. Manganese binding and oxidation were measured in incubation experiments using $^{54}Mn(II)$ as a radiotracer and filtering samples at specific times to distinguish particulate (bound and oxidized) ^{54}Mn from soluble (primarily Mn^{2+}) ^{54}Mn. $^{54}Mn(II)$ binding was measured in 10 ml of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (10 mM; pH 7.4) containing 50 μM $MnCl_2$, 1 μCi of $^{54}MnCl_2$ (specific activity, 50 μCi/μg of Mn), and various concentrations of $CuSO_4 \cdot 5H_2O$ (0 to 50 μM). The measurements were started by adding SG-1 spores (purified as described by Rosson and Nealson [58]) to a final concentration of 6.5×10^5 spores per ml. After incubating for 24 h at room temperature, replicate samples were taken for measurement of total ^{54}Mn and particulate ^{54}Mn. Duplicate 1-ml unfiltered subsamples were placed directly into gamma vials for counting total radioactivity in the subsample. For the particulate ^{54}Mn measurements, duplicate 1-ml samples were filtered onto 0.22-μm-pore-size membrane filters (Gelman; Supor 200) and washed with 2 ml of 10 mM HEPES (pH 7.4). The filters were placed in the bottom of a gamma vial, and 1 ml of 0.1% hydroxylamine-HCl was added to the filters to solubilize the oxidized ^{54}Mn and maintain similar counting geometry with the subsamples taken for total counts. The radioactivities of the filters and the total subsamples were counted for 5 min by using an LKB Compugamma CS 1282 gamma counter equipped with a 3-in. (7.62-cm) NaI well detector, and the percentage of ^{54}Mn in the particulate phase and its corresponding concentration were calculated. The amount of ^{54}Mn bound to the filters after washing is essentially the amount that has been oxidized (58). The Mn(II) binding (oxidation) rate was calculated assuming linear binding over the experimental time course.

Nucleotide sequence accession number. The nucleotide sequence of *mnxA* to *mnxG* and flanking DNA has been deposited in GenBank under nucleotide accession number U31081.

RESULTS

Nucleotide sequence analysis. The results of transposon mutagenesis experiments had implicated two chromosomal regions, Mnx I and Mnx II, as necessary for manganese oxidation by the spores (71). Abbreviated restriction maps of these regions are shown in Fig. 1. The ~5-kbp area between the *Hin*dIII and *Nde*I sites in Mnx I was sequenced, as was the ~1-kbp area within and slightly beyond the smaller *Nde*I-*Bam*HI fragment in Mnx II. The sequence from a clone from a transposon mutant (pLVM7E) that carried chromosomal DNA flanking the last insertion in the Mnx I region showed that the two regions were linked by a 250-bp *Nde*I fragment. This intervening sequence was confirmed by sequencing of PCR-amplified DNA from wild-type SG-1 chromosomal DNA. The complete nucleotide sequence of the area is presented in Fig. 2.

A total of seven possible ORFs, designated *mnxA* to *mnxG*, were identified, with a small portion of the end of an ORF at the beginning of the region presumably unrelated to manganese oxidation (Fig. 1 and 2). Approximate positioning of the transposon insertions within the regions based on previous restriction mapping data (71) places insertions in *mnxB*, *-C*, *-D*,

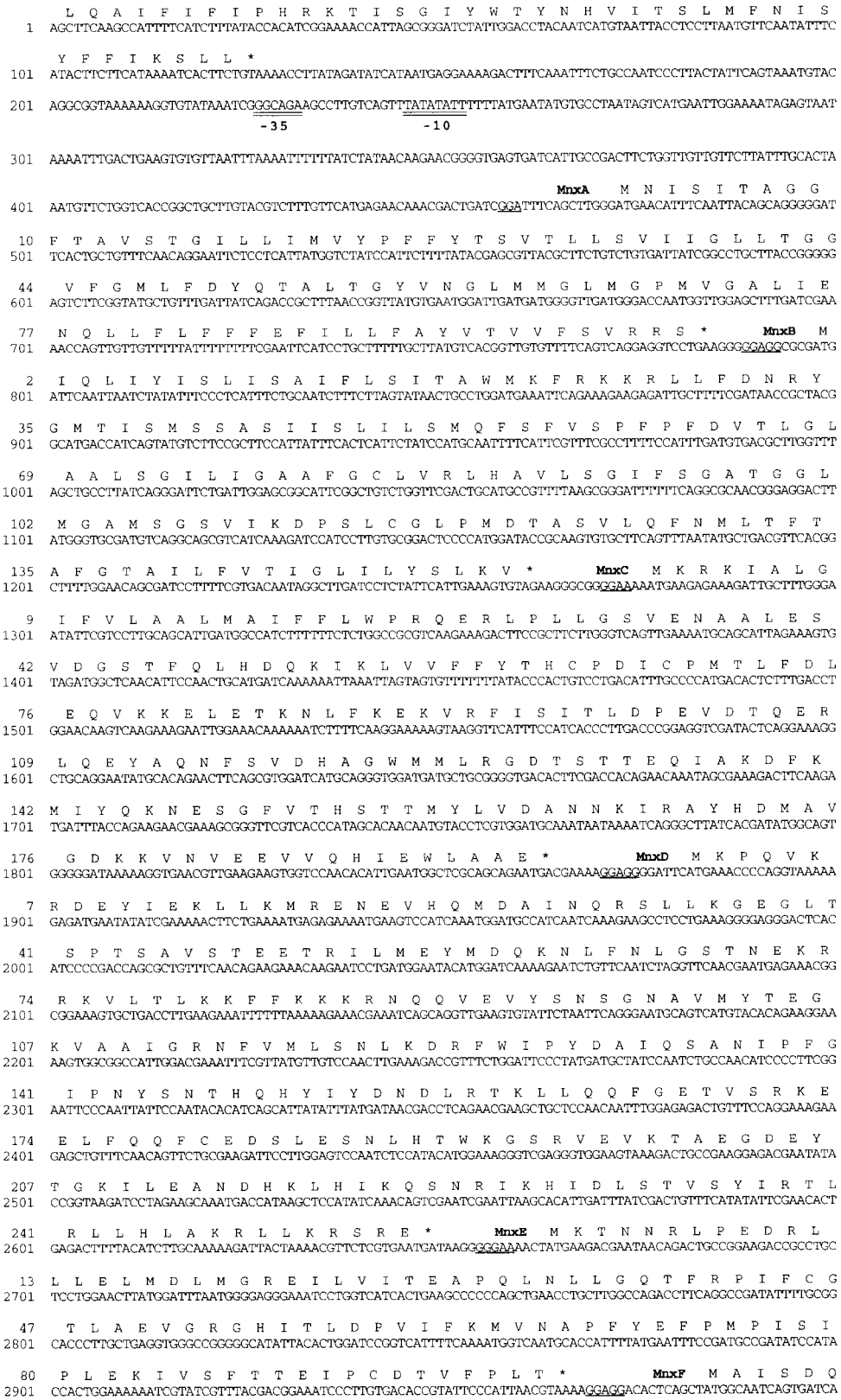

FIG. 2. Nucleotide sequence of the area involved in manganese oxidation that was sequenced in this study (numbered starting at 1 at the *Hin*dIII site). The predicted amino acid sequences of the seven ORFs within the region (plus a small portion of the end of an ORF at the beginning of the region) are shown in single-letter code above the nucleotide sequence. The start of each ORF was designated by the cooccurrence of a methionine preceded by an apparent Shine-Dalgarno sequence. The putative Shine-Dalgarno sequences are underlined, and putative −35 and −10 sequences are double underlined. Stop codons are indicated by asterisks.

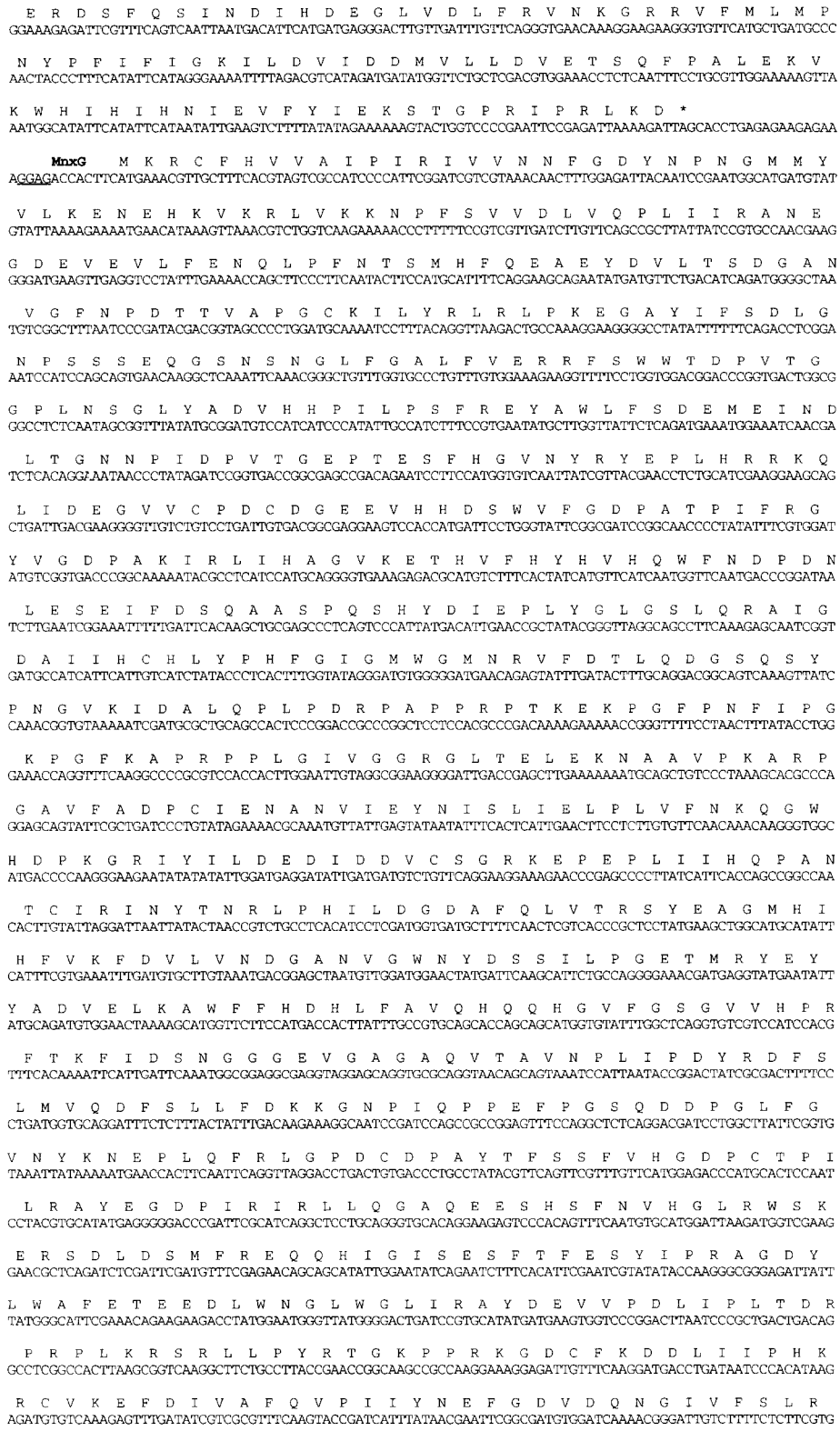

FIG. 2—*Continued.*

and -*G*. The *mnxA* to *mnxG* ORFs were preceded by a stretch of 344 nucleotides of noncoding sequence, and they were all oriented in the same direction. The close sequential arrangement of the genes, their similar orientation, and the fact that transposon insertions into most of the genes blocked the same function (manganese oxidation) suggest that the genes reside in an operon and are cotranscribed. Upstream of the translational start site of the first gene in the series (*mnxA*) are pu-

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 863  E D M D D I L S G L K N P E P L I I R A N V G D E V R V K L T S L L
5901  AGGACATGGATGATATTCTTTCAGGCCTTAAAAACCCCGAACCGCTTATTATCAGGGCCAATGTGGGGGACGAAGTCAGGGTAAAATTGACCAGCCTGCT

 897    E F D K F P F K D G I Y P Y P T V K E Q A F Y P P S L R I S L H P
6001  GGAGTTTGATAAGTTTCCTTTCAAGGACGGCATATATCCTTATCCTACAGTTAAGGAACAAGCATTTTATCCGCCGTCTTTAAGAATTTCCCTGCACCCT

 930  Q L I Q Y D V K T S S G E T V G Y N Y D Q T V G P G E S I T Y K W
6101  CAGTTGATTCAATATGATGTCAAGACTTCGAGTGGAGAAACCGTCGGATATAACTATGATCAGACGGTCGGTCCTGGCGAATCCATTACGTATAAATGGT

 963  Y V D F P V G A C G M W D M A D I R N H K S Q G A F G A F I A E P R
6201  ATGTCGATTTCCCTGTTGGAGCATGCGGCATGTGGGATATGGCCGATATCCGCAACCATAAATCACAGGGGGCCTTCGGTGCATTCATTGCTGAACCAAG

 997    G T E Y L D P H T L K P V K T G A S V V L R N N L L P D K R E F V
6301  GGGCACCGAGTATCTCGACCCACATACACTGAAGCCTGTCAAAACAGGTGCGAGTGTCGTGCTGAGAAACAACCTGCTGCCGGACAAAAGGGAATTTGTC

1030  I I M H D G V R L H D K D G I L I T D P I D G I L L G P D N P F E
6401  ATAATCATGCATGACGGGGTAAGGCTTCATGACAAGGACGGTATCCTCATCACCGATCCGATTGATGGAATCCTCCTCGGTCCGGATAATCCGTTTGAAG

1063  G D L V D T Y D N G S R G F N Y R S E R L I N R Y R K H P V L H D L
6501  GAGATTTGGTCGATACCTATGATAACGGGTCCAGAGGCTTTAACTATCGTTCCGAGAGATTGATCAACCGTTACCGAAAACATCCTGTACTTCATGATTT

1097    F S S K L F G D P S T P L F E S Y A G D P V T L R L I A P A E R R
6601  GTTCAGTTCGAAGCTATTCGGTGATCCGTCAACACCACTATTTGAAAGCTATGCAGGGGATCCGGTCACCCTCAGGTTAATTGCTCCTGCAGAAAGAAGA

1130  R S H T F H L H G H Y W R K D I S D I N S V T A S F E G F N L A G
6701  AGATCGCATACCTTCCATCTCCACGGACATTACTGGAGAAAAGATATCTCGGACATTAACTCTGTGACAGCTTCGTTTGAAGGGTTTAATCTTGCGGGAA

1163  S K Q D F E L F G G A G G F N Q F P G D Y M Y R S G N I Q W D I E Q
6801  GCAAACAGGATTTTGAATTGTTTGGAGGTGCTGGAGGTTTCAACCAATTCCCCGGCGACTATATGTATAGATCCGGAAATATACAGTGGGATATAGAACA

1197    G M W G I M R V H G K R Q P H L P P L E K *
6901  GGGGATGTGGGGAATCATGCGCGTGCACGGGAAGCGCCAGCCGCATCTTCCGCCTTTGGAAAAATAAAAAGGTGGTGGCACAATGGGAGCCAATGAGGAA

7001  CAAGACGGAAAGCAAAATGAGCTGGAGAAGAAAAAATCC
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FIG. 2—*Continued.*

tative −35 and −10 sequences, GGCAGA and TATATATT, that correspond with only one mismatch each to the mother-cell-specific σ^K −35 and −10 consensus promoter region sequences from *B. subtilis* (43), GKMACA and CATANNNT, respectively (Fig. 2). No other obvious transcriptional control elements were found in this upstream region, including any sequence resembling either a *B. subtilis* σ^E-type promoter sequence (43, 57) or a binding site (75) for GerE (the factor that regulates transcription from σ^K promoters in *B. subtilis* [77]).

Analysis of the predicted amino acid sequences. The deduced amino acid sequence for each of the ORFs identified is shown above the nucleotide sequence for the region in Fig. 2. A summary of the derived proteins is given in Table 2. The first ORF, *mnxA*, is a 312-bp stretch of nucleotides that encodes a putative polypeptide 103 amino acids in length with a deduced molecular mass of 11,275 Da. A gap of only 13 nucleotides separates *mnxA* from the next ORF, *mnxB*. MnxB is encoded by 465 nucleotides, making it 154 amino acids long, with a predicted molecular mass of 16,464 Da. These first two deduced proteins have 29% identity and are 70% similar when taking into account conservative substitutions (alignment score by the program RDF2 was 7.1 standard deviations above the mean; alignment not shown). Both deduced proteins are extremely hydrophobic. MnxA is 15.5% leucine, 11.6% phenylalanine, and 10.7% valine, and MnxB is 14.9% leucine and 11% isoleucine. The predicted isoelectric point of MnxB is basic (pI = 9.9).

The *mnxC* ORF is separated from *mnxB* by 14 nucleotides. MnxC consists of 195 amino acids, encoded by 588 bp, and has a predicted molecular mass of 22,549 Da. The majority of the deduced protein is hydrophilic except for a predominantly hydrophobic stretch of approximately 30 amino acids near the start that has characteristics of a bacterial signal sequence (51). The presence of a signal sequence suggests that *mnxC* may encode a protein that is targeted to the cell membrane or perhaps to the forespore membrane. What role such a protein would play in either sporulation or manganese oxidation by the spores remains unknown. The hydrophobic stretch has significant similarity (62% identity in 16 amino acids) to a small

TABLE 2. Summary of features of the deduced protein products of the *mnx* genes

Protein name	Length (amino acids)	Predicted molecular mass (kDa)	Feature(s)
MnxA	103	11	Highly hydrophobic; sequence similarity to MnxB
MnxB	154	16	Highly hydrophobic; sequence similarity to MnxA
MnxC	195	22	Sequence similarity to several cell surface or oxidoreductase-associated proteins (see Table 3)
MnxD	255	30	Gene expressed at stage IV of sporulation
MnxE	100	11	Highly hydrophobic
MnxF	99	12	
MnxG	1,218	138	Sequence similarity to family of multicopper oxidases

TABLE 3. Comparison of MnxC with similar proteins

Species and protein	% Identity/ length (aa)[a]	SD above random[b]
Cowdria ruminantium immunodominant protein	28.3/138	11.4
Anaplasma marginale MSP 5	25.7/140	8.7
Pseudomonas stutzeri ORF193	25.0/148	15.1
Staphylococcus aureus mer operon 18-kDa protein	27.3/88	8.3
Saccharomyces cerevisiae		
SCO2	26.9/160	14.0
SCO1	25.8/159	20.9

[a] The percentage of amino acid identity over the stretch of matching amino acids (aa) was determined from comparisons performed with the program FASTA.

[b] The results of shuffling analysis by use of the program RDF reported as the standard deviation above the mean for random alignments.

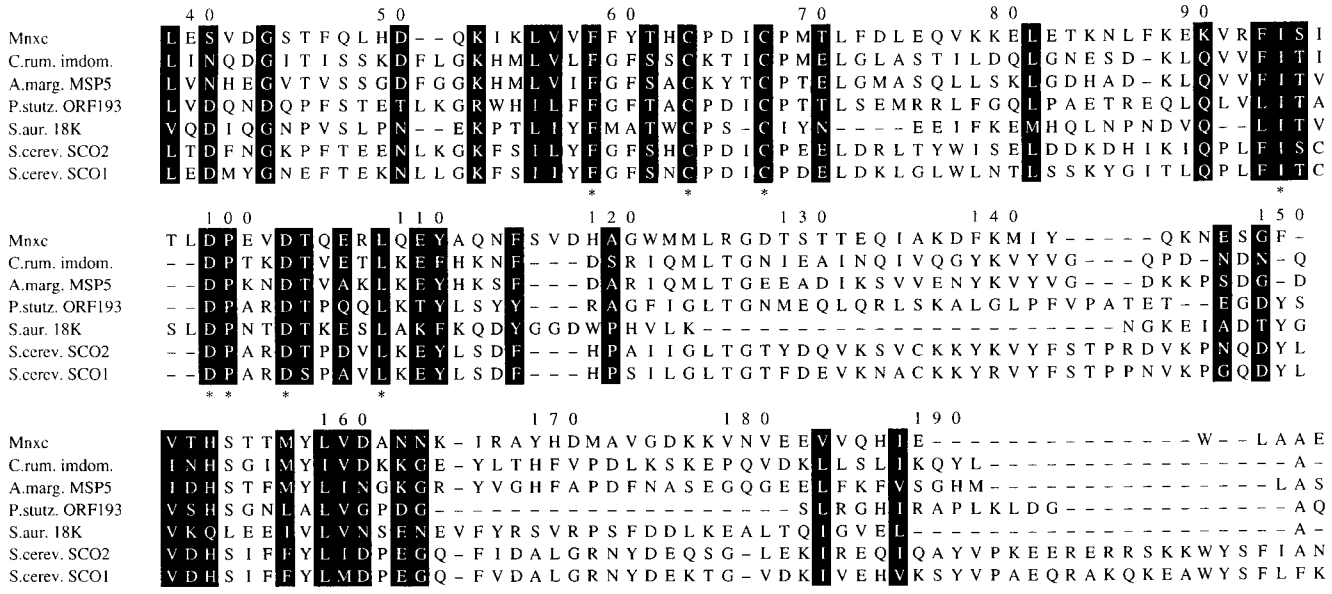

FIG. 3. Multiple alignment of MnxC with the other proteins of significant similarity by use of CLUSTAL. Shaded amino acids represent identical residues (marked by asterisks) or conservative replacements (PAM250 matrix). Abbreviations and Genbank accession numbers: C.rum. imdom., the immunodominant protein from *Cowdria ruminantium* (L07385); A.marg. MSP5, a major surface protein from *Anaplasma marginale* (S50594); P.stutz. ORF193, an ORF from *Pseudomonas stutzeri* (PSFNRAAPT [cds3]); S.aur. 18K, an 18-kDa protein from the *Staphylococcus aureus mer* operon (B29504); S.cerev. SCO1 (P23833) and SCO2 (P38072), proteins from *Saccharomyces cerevisiae*.

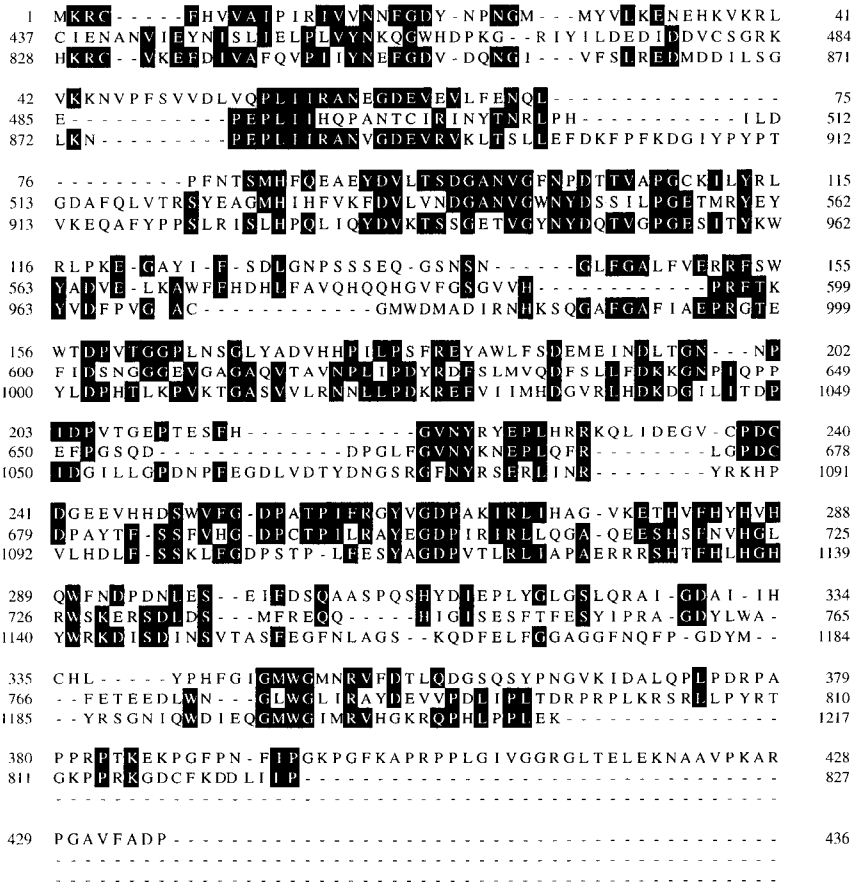

FIG. 4. Alignment of homologous domains within the amino acid sequence of MnxG. Identical amino acids present at the same site in two or more domains are shaded. Percentages of amino acid identity between domains are as follows: between the first and second, 23%; between the first and third, 27%; between the second and third, 23%.

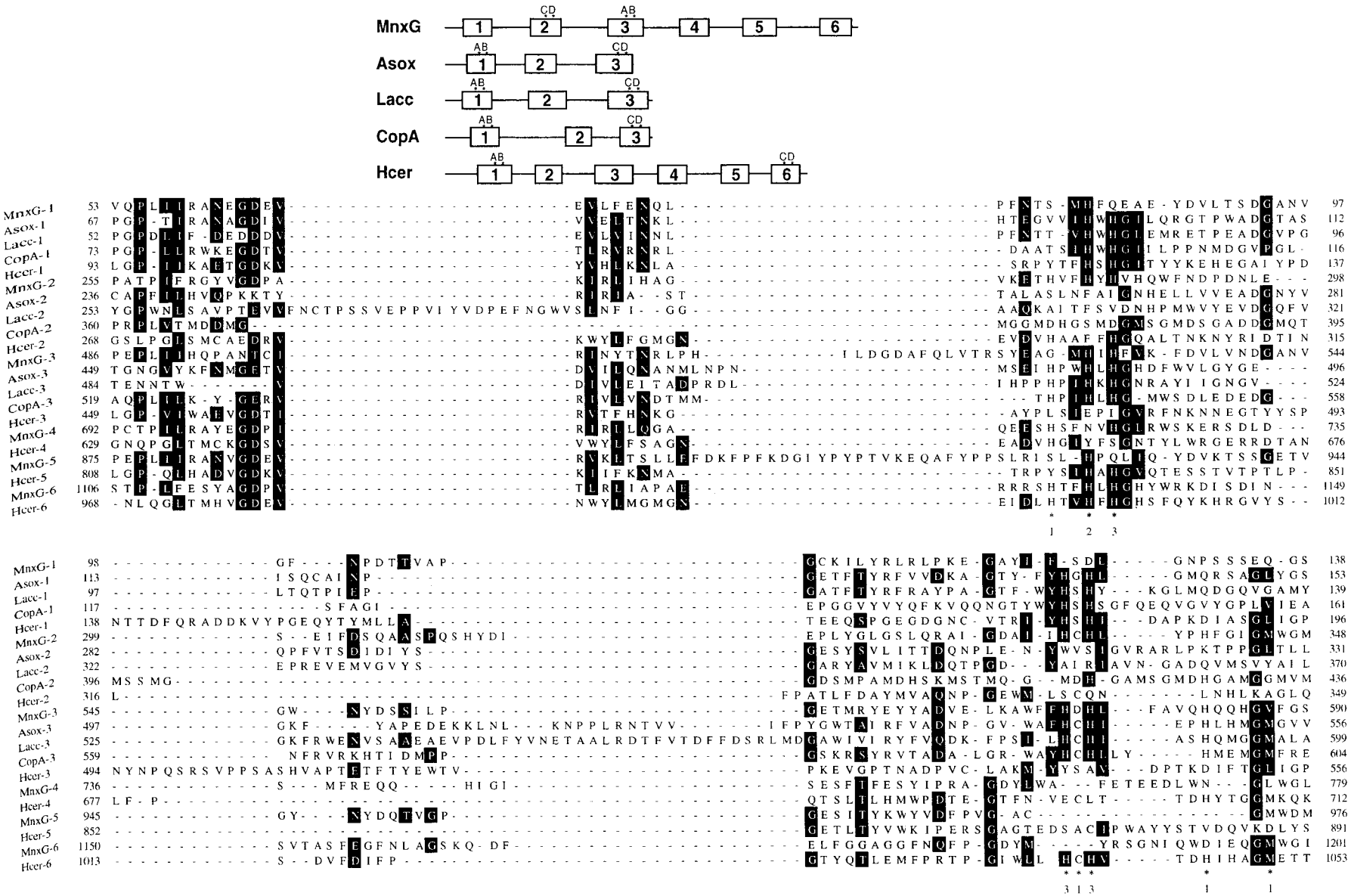

FIG. 5. Alignment of the amino acid sequences of portions of MnxG and portions of four members of the family of multicopper oxidases (conforming to reference 60). In the alignment, the numbers after the protein designation in each case refer to the protein domain in which that stretch of sequence resides. The diagram at the top of the figure shows the approximate location within each protein of the stretches of sequence included in the alignment (boxes) along with the corresponding domain number. The locations of the possible copper-binding motifs are designated by letters A, B, C, and D and correspond to the regions shown in Fig. 6A to D. In the sequence alignment, the amino acid residues conserved or identical with MnxG among 50% or more of the sequences are shaded. The residues thought to be the sites of copper binding in some of the domains of the multicopper oxidases are indicated by asterisks, and the number indicates which of the three types of copper (type 1, 2, or 3) is potentially bound to that particular residue (according to reference 60). Abbreviations and GenBank accession numbers: Asox, ascorbate oxidase from cucumber (J04494); Lacc, laccase from *Aspergillus nidulans* (X52552); Copa, CopA copper resistance protein from *Pseudomonas syringae* (M19930); Hcer, human ceruloplasmin (M13699).

hydrophobic region in the middle of the MerT protein of *Serratia marcescens* (49). The importance of this similarity is unknown, although it may be related to the similarity of MnxC to another protein in the *mer* operon (see immediately below). The remainder of the predicted amino acid sequence of the *mnxC* ORF (residue 39 to the carboxyl end) has significant similarity to six other proteins in the NCBI database (Table 3 and Fig. 3): an immunodominant protein from *Cowdria ruminantium* (37); a major surface protein (MSP5) from *Anaplasma marginale* (72); a partial ORF (ORF193; EMBL accession number S37399) located downstream of an *fnr*-like gene in *Pseudomonas stutzeri*; an 18-kDa protein from the *Staphylococcus aureus mer* operon (33); and two proteins from *Saccharomyces cerevisiae*, SCO1 (64) and SCO2 (EMBL accession number Z35893; recognized as having sequence similarity to SCO1). A seventh, recently discovered gene, *prrC* (GenBank accession number U22347), also shows similarity to this latter part of MnxC (data not shown). This gene, which is somehow involved in the regulation of photosynthesis gene expression in *Rhodobacter sphaeroides*, has been found to have sequence similarity to the SCO1 and SCO2 proteins mentioned above (21).

These proteins are found in diverse organisms, and the functional connections among them are not entirely clear. The *C. ruminantium* and *Anaplasma marginale* proteins are similar proteins and are recognized as major cell surface proteins of these rickettsial species. Two proteins are associated with multicomponent oxidoreductase systems: the 18-kDa *Staphylococcus aureus* protein is one of several oxidoreductases encoded by an operon involved in mercury resistance, and SCO1 is believed to be required for the assembly of the cytochrome *c* oxidase complex (64). In light of the involvement of the *mnx* genes in manganese oxidation, this information suggests that MnxC may be part of a larger multicomponent oxidoreductase system, perhaps with other proteins encoded in the Mnx region. The region of similarity in Fig. 3 includes two conserved cysteines at MnxC residues 64 and 68. Interestingly, the 18-kDa *Staphylococcus aureus* protein has been identified as having a thioredoxin motif which includes these two cysteines (20). Despite the presence of an additional amino acid between the cysteines in MnxC (Fig. 3), the secondary structure of MnxC in this region matches very well that of the thioredoxin motif with the two cystines residing in a turn, flanked by a β sheet and an alpha helix that is followed by an additional β sheet (20). This suggests that these cysteines in MnxC may have redox activity, and by analogy to thioredoxin, it is possible that the *mnxC* protein product may be a protein disulfide oxidoreductase. Separated from *mnxC* by 17 nucleotides is *mnxD*, which codes for a deduced protein of 255 amino acids with a calculated molecular mass of 29,898 Da. MnxD has a basic isoelectric point, pI 9.5, and is predominantly hydrophilic.

The *mnxE* ORF is 14 nucleotides downstream of *mnxD*. Its deduced protein is 100 amino acids in length (303 nucleotides) and has a predicted molecular mass of 11,333 Da. MnxE is very hydrophobic for the last four-fifths of its residues, with a total of 14% leucine, 10% phenylalanine, and 9% isoleucine residues. The *mnxF* ORF is 17 nucleotides downstream. Its predicted product is composed of 99 amino acids (300 nucleotides in length) and is predominantly hydrophilic, with a calculated molecular mass of 11,656 Da. A gap of 30 nucleotides separates *mnxF* from the last ORF in the cluster, *mnxG*.

The *mnxG* ORF is extremely large in comparison to the other *mnx* ORFs. It is capable of producing a protein that is 1,218 amino acids in length (3,654 nucleotides) and predicted to be 138 kDa in size. MnxG appeared to have internal repeating sequences and was analyzed for internal homology by use of the program DOTPLOT. Three large internal homology domains were discovered (Fig. 4).

Similarity of MnxG to the family of multicopper oxidases. Use of both the BLAST and FASTA (55, 56) database searching programs revealed stretches of amino acid sequence similarity between MnxG and various members of the multicopper oxidase family (60). In addition, searches against the protein motif library PROSITE identified in MnxG the signature region for copper binding in members of the family {H-C-H-X(3)-H-X-(3)-G-[L/M]; residues 334 to 345}.

The multicopper oxidase family is composed of distantly related members that contain conserved domains involved in binding multiple copper ions that are involved in electron transfer during the oxidation of various substrates (40, 52, 60). Multicopper oxidases bind copper ions of three spectroscopically distinct types, termed 1, 2, and 3. The copper 1 center accepts the electron from the substrate and shuttles it to the 2 and 3 centers, which are believed to bind and reduce molecular oxygen (30). The multicopper oxidase family includes three established members: laccase, isolated from the lacquer tree and from certain fungi; ascorbate oxidase, isolated from cucumber and squash; and ceruloplasmin (also called ferroxidase), a protein isolated from vertebrate plasma that can oxidize a number of substrates, including iron(II). A member recently included in the family is a plasmid-encoded protein from *Pseudomonas* and *Xanthomonas* species (*copA*) which is involved in copper resistance (35, 39). Other members of the family include the blood coagulation factors Fa V and Fa VIII, which lack oxidase activity but retain sequence similarity to the family (60).

On the basis of internal homologies of the copper oxidases, a sequence alignment was made between regions of MnxG and the copper oxidases, a portion of which can be seen in Fig. 5. Ascorbate oxidase contains three homologous domains, as does laccase, while ceruloplasmin is composed of three larger homology regions, each of which shows signs of internal duplication (40). Ryden and Hunt (60) recently analyzed the blue copper-containing oxidases and related proteins and made an alignment of the amino acid sequences of the proteins on the basis of their domain structure. The Ryden and Hunt alignment was used as a template in our alignment, except that a laccase more similar to MnxG was used, and CopA was added and aligned on the basis of its similarity to ascorbate oxidase. Regions of MnxG were aligned with regions of the copper oxidases that appeared similar on the basis of FASTA and BLAST database search output, and refinements were made by eye. The portion of the alignment that showed the highest similarity between MnxG and the copper oxidases is shown in Fig. 5. This is approximately the latter half of the alignment and includes all of the sites in the copper oxidases believed to be involved in copper binding. FASTA and BLAST results suggested that the three homologous domains of MnxG may each have an internal duplication, much like ceruloplasmin, and therefore a six-domain structure of MnxG (from here on called regions 1 to 6 and designated MnxG-1 through MnxG-6) was used in the alignment (Fig. 5). A statistical comparison, using the program ALIGN of the pairwise alignment scores of MnxG and the copper oxidases to that of a randomization of the sequences, demonstrated that several of the MnxG regions matched significantly the copper oxidases. Some of the strongest similarities were as follows: between MnxG-1 (residues 53 to 138) and laccase (Lacc-1; residues 52 to 139), 25% identity (a score of 9.1 standard deviations above the randomized mean); between MnxG-1 (residues 42 to 160) and ascorbate oxidase (Asox-1; residues 55 to 185), 22% identity (a score of 7.1); between MnxG-3 (residues 486 to 590) and CopA

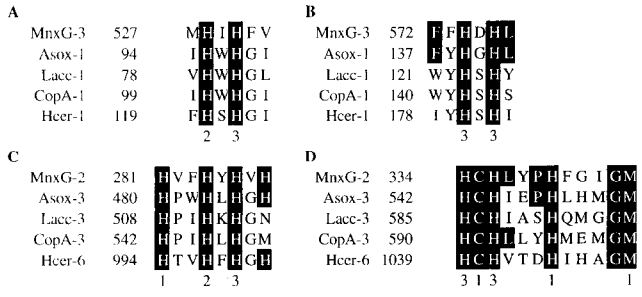

FIG. 6. Alignment of the amino acid sequences of areas in the multicopper oxidases believed to be involved in copper binding (conforming to reference 50) with proposed copper-binding sites in MnxG. The amino acid residues identical to MnxG are shaded, and the copper-binding residues are numbered according to the type of copper they potentially help coordinate. Abbreviations are as described in the legend to Fig. 5.

(CopA-3; residues 519 to 604), 30% identity (a score of 4.0); and between MnxG-2 (residues 255 to 348) and ceruloplasmin (Hcer-6; residues 968 to 1053), 26% identity (a score of 3.5), this last comparison being that which contains the copper oxidase signature motifs.

Putative copper-binding sites can be assigned to residues in MnxG on the basis of comparison with the copper oxidases (Fig. 5 and 6). The most obvious assignment is the signature region for the copper oxidase family (Fig. 6D) found in MnxG (region 2; residues 334 to 345) in the PROSITE database search (see above). Upstream of that area, within the same region of MnxG, as is the case in the copper oxidases, is another cluster of potential copper-binding residues (Fig. 6C). Two other clusters of potential copper binding residues (Fig. 6A and B) reside in region 3 of MnxG, corresponding to the two clusters that reside in the first domain of each of the copper oxidases. There is also a cluster of histidine residues in region 6 of MnxG (residues 1132 to 1139) that matches very well to a copper-binding site in ceruloplasmin (Fig. 6C). Therefore, this site may also be involved in copper binding, but a corresponding binding site like that in Fig. 6D was not found downstream within the same region of MnxG and so this site was not included as a potential binding site in Fig. 6. A prediction of secondary structure of MnxG indicated a high likelihood of β-strand structure around the proposed copper-binding sites and in a number of other sites that, on the basis of our alignment, correspond to the β-strand structure known to exist in ascorbate oxidase and believed to be conserved in the copper oxidases (40, 60).

Copper enhancement of manganese oxidation. The sequence similarities between regions of MnxG and the copper oxidases prompted us to investigate the effect exogenously added copper would have upon manganese oxidation by SG-1 spores by use of ^{54}Mn radiotracer techniques (Fig. 7). Addition of low amounts of Cu(II) enhanced the formation of particulate ^{54}Mn (primarily manganese oxide) by over a factor of two relative to no Cu(II) additions. The optimum copper concentration for Mn oxidation was ~1 μM. At higher Cu(II) concentrations, Mn oxidation was inhibited, perhaps because copper may begin to compete for an Mn-binding site on the protein, thus reducing Mn oxidation. The fact that copper enhances manganese oxidation suggests that a copper oxidase-like mechanism may be involved in Mn oxidation. When coupled with the similarity of MnxG to the copper oxidases, these results suggest that MnxG may be directly involved in Mn oxidation.

MnxD is expressed at stage IV to V of sporulation. The timing of expression of *mnxD*, the gene in which Tn*917*-LTV1 had inserted in LTM2, was examined during growth and sporulation by monitoring the activity of the transposon-encoded *lacZ* gene (Fig. 8). The activity commenced at around stage III of sporulation (sporulation stages described in Materials and Methods) and peaked between stages IV and V of sporulation, when the population contained about 50% phase-bright forespores.

Phenotypic characterization of spores from nonoxidizing mutants. The spores produced by the nonoxidizing mutants were tested for altered resistance properties (LTM2, -14, -18, -22, and SG-1W) and germination characteristics (all mutants) (see reference 48 for methods). All of the mutants produced phase-bright spores. The mutant spores were as resistant to heat (80°C for 10 min), lysozyme (250 μg/ml and incubation at 37°C for 10 min), and chloroform or ethanol (10-min exposure) as were wild-type spores. Likewise, no differences were seen in germination characteristics of the mutant spores when a tetrazolium dye overlay method was used (48).

The spores produced by the nonoxidizing mutants were also analyzed by transmission electron microscopy for differences in structure based on appearance (Fig. 9). Coats of SG-1 spores appear to consist of four distinct layers: a granular undercoat, a laminar inner spore coat, an electron-dense middle spore coat (that corresponds to the outer spore coat of *B. subtilis*), and an outermost, laminar spore coat that is present as ridges over the spore (68). Manganese is oxidized and precipitates on the outermost ridged structure. Examination of spores from several of the nonoxidizing mutants (LTM2, -7, -14, -18, -22, SG-1W, and SG-1WBT) suggested that there are differences in the outermost spore coat compared with that of the wild type. The outer coat often appeared to be sloughing or peeling off in layers from the spore surface in the nonoxidizing spores (Fig. 9), suggesting that its layers are poorly cross-linked. All of the mutants examined appeared to be disrupted in the outer spore coat, but no obvious differences in the severity or the exact nature of the disruption could be distinguished among the mutants. No differences were seen in any other part of the spore, including the inner spore coat, the middle spore coat, or in the cortex. It should be mentioned that conclusions based on mutant analysis may not apply to *mnxA* since this gene may not be affected by any of the mutations.

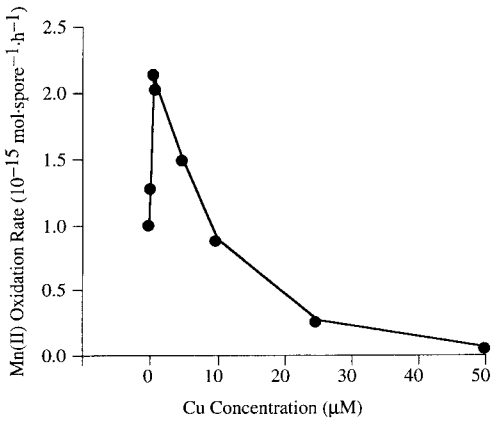

FIG. 7. Effect of the addition of copper on the rate of manganese oxidation by SG-1 spores.

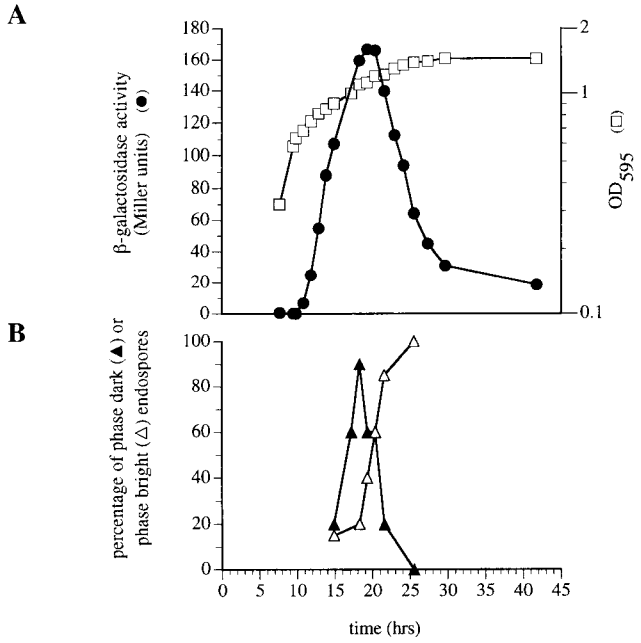

FIG. 8. Transcription of the Tn*917-lacZ* fusion in the LTM2 nonoxidizing mutant. (A) The specific activity of β-galactosidase (closed circles) was determined during growth and sporulation of LTM2. Sporulation was induced by nutrient exhaustion following growth in K medium. Growth was monitored by optical density at 595 (OD_{595}) (open squares). Sporulation begins late in the exponential growth phase (at approximately t = 8 h of growth). The small amount of endogenous β-galactosidase activity present in wild-type SG-1 (1.8 Miller units) has been subtracted from each sample. (B) The corresponding approximate percentages of phase-dark forespores (closed triangles) and phase-bright endospores (open triangles) present in the population during sporulation as determined by phase-contrast microscopy are shown. The approximate timing (in hours after inoculation) of the stages of sporulation (as defined by the presence of certain cell types as described in Materials and Methods) is as follows: stage III, t = 10.5 h; stage IV to VI, t = 13 h onward.

DISCUSSION

In this article, we present nucleotide sequence analysis and characterization of genes involved in manganese oxidation by spores of SG-1. We speculate that perhaps some of the genes encode spore coat proteins. The regions were first identified because transposon insertions disrupted a spore surface-associated phenomenon, manganese oxidation (17, 71). A mother cell sigma factor (σ^K) recognition sequence similar to that found in *B. subtilis* was found upstream of the region, and expression of many spore coat protein genes is known to be under the control of this sigma factor (65). Expression of *mnxD*, as monitored by the β-galactosidase activity of a Tn*917-lacZ* insertion within the gene, begins in stage III and peaks in mid-sporulation to late sporulation at stages IV to V. If the *mnx* genes are indeed cotranscribed, as their operon-like organization suggests, then their products would all be produced at that time. This expression commences slightly earlier than the expression seen from σ^K-directed spore coat protein genes of *B. subtilis*, which begin activity at around t_4 (stage IV) (61, 63), but somewhat later than the σ^E-directed coat genes, whose activity begins at t_2 (stage II) (26, 78). Transmission electron microscopy of the nonoxidizing mutant spores indicates alterations in the outermost spore coat, suggesting that these genes may represent components of the outermost spore coat or proteins involved in the deposition of that structure. The fact that insertions in these regions did not affect resistance or germination properties of the spores should not be interpreted as indications that individual spore coat components have not been disrupted, since many spore coat mutants exhibit no alterations in their phenotype, presumably because of redundancies built into the spore coat structure (65).

It is possible that the region we have identified includes the oxidizing factor itself, presumably a spore coat protein, most likely residing in the outermost spore coat layers in SG-1. MnxG is a good candidate for the oxidizing factor. MnxG is a large protein (predicted to be 138 kDa), and the manganese-oxidizing factor that has occasionally been identified in protein gels has a high molecular mass (205 kDa) (59). The discrepancy in size may be due to cross-linking of MnxG with itself or other spore coat components or possibly to some other modification of MnxG. Regions of significant sequence similarity were found between MnxG and the copper oxidases, including the copper-binding regions, suggesting that MnxG may function as a copper oxidase. Since the addition of copper at low concentrations was found to enhance manganese oxidation, a possible connection between MnxG and manganese oxidation is inferred. It is interesting to note that laccase, an enzyme commonly found in white rot fungi, which is one of the copper oxidases to which MnxG is similar, is capable of catalyzing one-electron oxidation reactions that produce Mn(III) chelates from Mn(II) (3).

It is possible that MnxG is the only gene product in this putative operon directly involved in manganese oxidation, since insertions upstream of *mnxG* may be exerting a polar effect on *mnxG*. However, the possibility also exists that one or more of the other Mnx proteins are also required for activity. This is supported by the sequence similarity of MnxC to proteins involved in multicomponent oxidoreductase systems, suggesting that MnxC and MnxG may also be part of such a system.

It was recently discovered that the putative manganese-oxidizing protein from *Leptothrix discophora*, MofA (GenBank accession number Z25774) (11), has similarity to two apparent additions to the copper oxidase family, phenoxazinone synthase (29, 66) and bilirubin oxidase (32). Additionally, we have found that MofA has similarity to the copper oxidase CopA (as did MnxG) and to a bacterial cell-division-related gene, *sufI* (10), that does not contain copper-binding motifs but appears to have other sequence similarity to the multicopper oxidases (data not shown). Regions of similarity between MofA and the multicopper oxidases include two of the four copper-binding motifs (Fig. 6A and B but not C and D) (29, 66). MofA appears to have other histidine residues present that would complete the type 2 and type 3 copper centers but appears to lack a full complement of type 1 copper-binding residues (data not shown). Since the role of type 1 copper centers is the initial oxidation of the substrate, and manganese oxidation in *L. discophora* can be catalyzed by a single protein (as determined by SDS-polyacrylamide gel electrophoresis) (1, 6) which appears to be MofA, the initial oxidation step in MofA may be by a somewhat different mechanism than that found in the copper oxidases. MnxG, on the other hand, contains all three copper centers and therefore may carry out both the initial oxidation step and the binding and reduction of molecular oxygen in a manner similar to that of a copper oxidase. Although no significant overall sequence similarity was found between MnxG and MofA, the fact that they both have similarity to copper oxidases and contain copper-binding motifs suggests that the use of copper as a cofactor in manganese-oxidizing proteins may be a common theme.

The function of manganese oxidation by SG-1 spores still remains to be discovered. Since vegetative cells of SG-1 are known to reduce manganese oxide, the manganese oxide en-

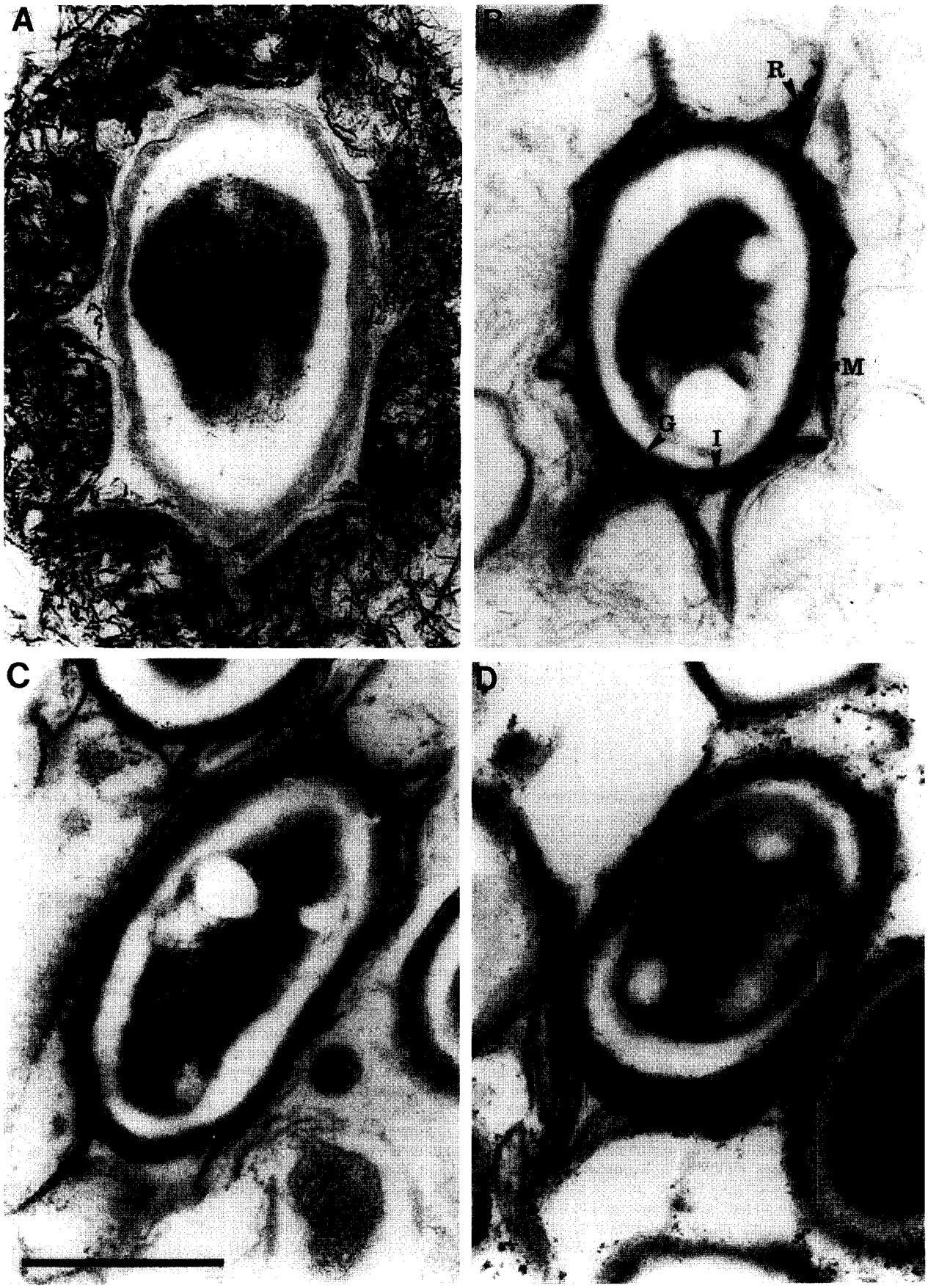
A
R
M
G
I
C
D

FIG. 9. Transmission electron microscopy of spores of SG-1. (A) SG-1 spores encrusted with manganese oxide (from reference 68). (B) SG-1 spores grown without extra manganese(II) added to the medium and therefore not encrusted with manganese oxide, showing the laminar structure of the ridged, outermost spore coat on which manganese oxide precipitates (the spore coat structures are labeled: G, granular undercoat; I, inner spore coat; M, middle spore coat; R, outermost, ridged spore coat). (C and D) Spores of nonoxidizing mutants LTM22 and LTM18, respectively, in which the outermost spore coat appears to be poorly cemented to the surface compared with that of the wild type. Bar, 0.5 μm.

crusting the spores could be used as an electron acceptor for anaerobic growth upon germination of the spores (17, 68). Another possibility relates to the suggestion that the *B. subtilis* spore coat protein CotE may have peroxidase activity (15) and that this activity may be linked (66) to its possible involvement in cross-linking of the spore coat (76). By analogy, the function of MnxG may be to cross-link the outermost spore coat and additionally confer Mn oxidation activity upon the mature spore. This explanation is supported by the fact that the outermost spore coat seems to be disrupted in the nonoxidizing mutants.

The information obtained in this study should be extremely valuable in our further investigation of the phenomenon of manganese oxidation in SG-1 as well as our understanding of spore coat structure and assembly in *Bacillus* species. We will now be able to examine whether spore coat proteins of the molecular weight predicted by these ORFs are absent in various mutants. In addition, the identification of these genes allows us to initiate a detailed complementation analysis, which may result in a definitive determination of whether MnxG is the sole manganese-oxidizing factor or whether other Mnx proteins are involved. Finally, we can use the cloned genes to express and purify the Mnx proteins for use in directly testing for manganese oxidation activity (in the case of MnxG) and for generating specific antibodies against the individual proteins. These experiments should greatly aid in determining where the protein products of the *mnx* genes are localized and what roles the Mnx proteins play in manganese oxidation.

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