Genetic Analysis of the Marine Manganese-Oxidizing *Bacillus* sp. Strain SG-1: Protoplast Transformation, Tn*917* Mutagenesis, and Identification of Chromosomal Loci Involved in Manganese Oxidation

LORRAINE G. VAN WAASBERGEN,[1]† JAMES A. HOCH,[2] AND BRADLEY M. TEBO[1]*

Marine Biology Research Division, Scripps Institution of Oceanography, University of California—San Diego, La Jolla, California 92093,[1] and Division of Cellular Biology, Department of Molecular and Experimental Medicine, Research Institute of Scripps Clinic, La Jolla, California 92037[2]

Mature spores of the marine *Bacillus* sp. strain SG-1 bind and oxidize manganese(II), thereby becoming encrusted with a manganese(IV) oxide. Both the function and mechanism of this oxidation are unknown, although evidence suggests that spore coat proteins are involved. To further study this phenomenon, methods of genetic analysis were developed for SG-1. By a modified protoplast transformation procedure, SG-1 was transformed (~100 transformants per µg of DNA) with several different plasmids of gram-positive origin. Transposon Tn*917*, delivered on the temperature-sensitive plasmid pLTV1, was used to generate mutants of SG-1. Conditions were established that allowed 98% plasmid loss and insertions to be recovered at a frequency of 10^{-3}. Each mutant was found to be the result of a single insertion event. Restriction analysis of 27 mutants that do not oxidize manganese but still sporulate localized 17 of the insertions within two regions of the chromosome (termed Mnx regions), and a physical map of these regions was generated. Analysis of 18 transposon integrants in which manganese oxidation was unaffected revealed random transposon integration, with none of their insertions mapping within the Mnx regions. The Mnx regions were cloned from wild-type SG-1, and the largest region, carried on the lactococcal plasmid pGK13, was used to complement in *trans* one of the nonoxidizing mutants. These results demonstrate that the Mnx regions encode factors that are required for the oxidation of manganese, and this represents the first report identifying genes involved in bacterial manganese oxidation.

The biogeochemical cycling of manganese is recognized as an environmentally important process not only because manganese is an essential nutrient for all organisms but also because the oxidation and reduction of manganese are intimately coupled with the cycling of other essential elements. Field studies conducted over the past decade have firmly established that microbes play an important role in the natural cycling of manganese (15, 36). A diverse array of bacteria, fungi, and algae have been shown to catalyze the oxidation of manganese (18, 33). These organisms convert soluble manganese(II) to manganese(III) or manganese(IV), which generally precipitates around the cells as a brownish-black oxide or oxy-hydroxide. In some cases, the oxidation is known to be an indirect result of a rise in pH or E_h generated by the cell, while in other cases some component of the cell, either protein or polysaccharide, has been implicated in mediating the oxidation directly (35, 36).

The *Bacillus* sp. strain SG-1 was isolated from a marine sediment in a manganese enrichment culture (34). Dormant, mature spores of the organism catalyze the oxidation of manganese (22, 38). Kinetic studies of manganese oxidation (22) and examination of the spores by transmission electron microscopy (44) suggest that a component of the spore surface is binding and subsequently oxidizing the manganese. Isolated spore coats retain full oxidizing activity (13), and inhibitor

studies suggest that a protein is catalyzing the oxidation (38). From these studies, we can infer that a spore coat or exosporium protein is responsible for the oxidation, but we have been unable to isolate the oxidizing factor from the spores in sufficient quantities for analysis. It is not yet known what portion of the overall oxidation of manganese occurring in natural environments can be attributed to the activities of spores such as those produced by SG-1, but bacterial spores covered with manganese oxide have been observed in natural samples (17, 44), and we have been able to readily isolate manganese-oxidizing, spore-forming bacteria from marine sediments.

Spores from a variety of *Bacillus* species, including *Bacillus subtilis*, have been seen to gradually accumulate manganese oxide (18, 44, 47), but the precipitation of manganese by SG-1 spores is considerably more rapid than in most other cases. *B. subtilis* produces a spore coat protein (CotA) that imparts a brown color to sporulating colonies (14, 25, 40). This pigment is likely associated with manganese because it becomes darker as more manganese is provided in the medium (25). However, by testing with Leukoberbelin blue, a manganese oxide-specific dye (31), we have found that the pigment is not manganese oxide.

Many *Bacillus* species (1, 10, 16, 23) require elevated levels of manganese for sporulation. Furthermore, manganese, like other metal ions, is accumulated during mineralization of the spore coat (27, 32) and has been seen in some cases to enhance the heat resistance of bacterial endospores (1, 32). It is not known whether these phenomena are in any way related to manganese oxidation by spores of SG-1 or whether manganese

---

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Bacillus sp. strain SG-1		
Wild type		Laboratory collection (34)
LTM1 to LTM27	Tn*917* manganese oxidation (Mnx^a) mutants	This study
E. coli		
HB101	*hsdS20* ($r_B^- m_B^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44* F$^-$ λ^-	5
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lac^{q}ZΔM15* Tn*10* (Tc^r)]	Stratagene (7)
Plasmids		
pIL253	Em^r, gram-positive broad-host-range, structurally stable plasmid	42
pLTV1	Tn*917* delivery vehicle [on transposon portion, Em^r, promoterless *lacZ*, ColE1 replicon with Ap^r, on replicon portion, Tc^r, pE194(Ts) replicon]	P. Youngman (8)
pBR322	Ap^r, *E. coli* cloning vector	4
pSE380	Ap^r, *E. coli* cloning vector with super-linker region	Invitrogen Co. (6)
pGK13	Em^r Cm^r, broad-host-range cloning vector (a derivative of the lactococcal plasmid pWV01)	28
Recombinant plasmids		
pLVM7E, 14E, 17E, 24E, and 26E	Plasmids formed by self-ligating *Eco*RI-digested chromosomal DNA from SG1LTM7, 14, 17, 24, and 26, respectively, and transforming *E. coli* for Ap^r	This study
pBRM17N	8.6-kb *Nde*I, Mnx fragment from SG-1 cloned into pBR322	This study
pBRM26N	5.1-kb *Nde*I, Mnx fragment cloned into pBR322	This study
pSEM17N	8.6-kb *Nde*I, Mnx fragment cloned into pSE380	This study
pGKM17N	8.6-kb *Nde*I, Mnx fragment cloned into pGK13	This study

^{a} Mnx is the term applied to the phenotype of manganese oxidation distinct from the process of sporulation.

oxidation is linked to some other sporulation process. It has been suggested that because vegetative cells of SG-1 are able to reduce manganese oxide, presumably during anaerobic respiration (12), the accumulation of manganese oxide could perhaps be of some advantage upon germination of the spores (13, 44).

The goal of the present study was to develop genetic techniques with SG-1 in order to identify genes involved in manganese oxidation. Here, we report the development of a plasmid transformation system, transposon mutagenesis with Tn*917* (delivered on pLTV1 [8]), and the identification and analysis of chromosomal loci involved in the oxidation of manganese.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used or generated during this study are listed in Table 1. pLTV1 (8) carries a version of the *Streptococcus faecalis* transposon Tn*917*. The plasmid is highly temperature sensitive for replication (replication is normally blocked at temperatures higher than 37°C). Thus, the plasmid can be lost from cultures by growth at a relatively low temperature so that chromosomal insertions can be recovered. Tn*917*-LTV1 contains both a promoterless copy of the *lacZ* gene (oriented to create transcriptional fusions to the genes into which the transposon inserts) and a ColE1 replicon to facilitate cloning of genes flanking the insert.

Media and culture conditions. Agar (1.5% [wt/vol]; Bacto agar; Difco Laboratories]) was added for solid media unless otherwise noted. The sporulation medium for SG-1 is a modified K medium (38) containing 2.0 mg of peptone (Difco) per ml and 0.5 mg of yeast extract (Difco) per ml in artificial seawater (0.3 M NaCl, 0.05 M MgSO$_4$, 0.01 M CaCl$_2$, 0.01 M

KCl) with both 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.7]) and 100 μM MnCl$_2$ (filter sterilized) added after autoclaving. K$_R$ medium is a soft-agar regeneration medium used to plate protoplasts of SG-1 after protoplast transformation. K$_R$ medium is identical to K medium, except that to aid in stabilization of protoplasts during regeneration, it contains 0.8% (wt/vol) agar, 20 mM MgCl$_2$, and 0.01% (wt/vol) filter-sterilized bovine serum albumin (Sigma). The latter two components were added after autoclaving. R medium, used to grow SG-1 during transposon mutagenesis and for preparation of plasmid and chromosomal DNA, is similar but contains 10 mg of peptone per ml and 5 mg of yeast extract per ml, and manganese is omitted. SG-1 was grown at 30°C unless otherwise specified. For selection of plasmids carried by SG-1, erythromycin was used at 1 μg/ml and chloramphenicol was used at 10 μg/ml. Tetracycline was used at a relatively high concentration (60 μg/ml) to compensate for the high levels of magnesium (an antagonist of tetracycline) present in seawater in growth media. During transposon mutagenesis and for culture of transposon mutants, erythromycin was used at a concentration of 0.15 μg/ml, a concentration that is still selective against wild-type SG-1. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside for SG-1 was used in K agar medium at a concentration of 80 μg/ml. *Escherichia coli* was grown in Luria-Bertani medium (39) with ampicillin added at 50 μg/ml or erythromycin added at 100 μg/ml as appropriate. Restriction endonucleases and DNA-modification enzymes were used according to the manufacturer's specifications.

Isolation of plasmid DNA. Plasmid DNA was isolated from *E. coli* by alkaline lysis (39). Plasmids were isolated from *Bacillus* species (*B. subtilis* and *Bacillus* sp. strain SG-1) by a modified alkaline lysis procedure (29) with the following changes. Cultures were washed once with ice-cold TE buffer

(10 mM Tris-HCl, 1 mM EDTA [pH 7.6]) prior to lysis, and the cells were incubated in TES lysis buffer (50 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl [pH 8.0]) on ice for 30 min, followed by incubation for 15 min at 37°C. In addition, the incubation time on ice in the neutralization solution (3 M potassium acetate, pH adjusted to 4.8 with glacial acetic acid) was extended to 45 min. Plasmids could also be isolated from small-scale (5-ml) cultures of *Bacillus* cells with the Magic Minipreps DNA Purification System (Promega Corporation) modified in the following manner. The entire 5 ml was harvested by centrifugation (12,000 × g) at 4°C, washed with 1 ml of ice-cold TE buffer, and resuspended in 200 μl of Promega's Cell Resuspension Solution (50 mM Tris-HCl, 10 mM EDTA, 100 μg of RNase A per ml [pH 7.5]) supplemented with approximately 0.5 mg of lysozyme. The remainder of the procedure was followed according to the manufacturer's instructions.

Transformation of SG-1. A method for transforming SG-1 with plasmid DNA was developed by altering the protoplast transformation procedure used for *B. subtilis* (9). Modifications made to the procedure during its development are described in Results. The method is given here in its final form. All glassware used throughout the procedure was washed copiously with deionized water prior to sterilization to remove any traces of detergent that might cause lysis of the protoplasts. The buffer used throughout the majority of the procedure was (1/2S)MAK, which is an equal volume mixture of two solutions, (i) NS1 (nutrients plus seawater: 8.0 mg of peptone per ml, 2.0 mg of yeast extract per ml, 40 mM HEPES [pH 7.7], 200 μM MnCl$_2$ in 2× artificial seawater) and (ii) 2× (1/2S)MA (0.5 M sucrose, 0.04 M MgCl$_2$, 0.04 M malic acid [pH adjusted to 6.5 with NaOH]). Some experiments were performed in which (1/2S)MAK was replaced by a non-seawater-based buffer, SMA−, which is an equal volume mixture of N1 (NS1 with deionized water used in place of seawater) and 2× SMA (1.0 M sucrose, 0.04 M MgCl$_2$, 0.04 M malic acid [pH 6.5]).

A culture of SG-1 was grown at 30°C with vigorous shaking in K medium to late log phase. This culture was used to inoculate 1/100 a 50-ml culture of K medium grown at 30°C to an optical density at 660 nm of approximately 0.4 (4.4 × 10^8 cells per ml). Cells were harvested by centrifugation in a Sorvall HB-4 rotor (4,500 × g at 4°C for 15 min). Protoplasts of SG-1 were generated by gently resuspending the culture in 5 ml of (1/2S)MAK buffer (described above) containing 0.5 mg of lysozyme per ml and incubating the suspension at room temperature for approximately 45 min (until ~90% of the cells had protoplasted as observed microscopically). Protoplasts were centrifuged at 2,600 × g for 15 min at 4°C, washed one time without resuspension with 5 ml of (1/2S)MAK, recentrifuged, and resuspended in 5 ml of (1/2S)MAK. For transformation, 0.5 ml of washed protoplast suspension was added to 1 to 6 μg of plasmid DNA (purified by CsCl-gradient centrifugation) in TE buffer or water. To this mixture was added 1.5 ml of filter-sterilized 40% (wt/vol) polyethylene glycol (PEG [molecular weight, 1,000]) in 1× SMA buffer (see preceding paragraph). The suspension was mixed lightly and incubated for 2 min before dilution with 5 ml of (1/2S)MAK. The transformation mixture was pelleted at 2,600 × g at 4°C for 20 min and was resuspended in 1 ml of (1/2S)MAK. This suspension was incubated for 3 h at 30°C with shaking at ~100 rpm to allow recovery and expression of antibiotic resistance. Dilutions in (1/2S)MAK were spread on K$_R$ medium (see Media and culture conditions) or K$_R$ containing selective levels of the appropriate antibiotic and incubated in loosely closed bags at 30°C. The samples could also be concentrated prior to plating by centrifugation for 7 min at 7,200 × g at 4°C in a microcen-

trifuge. Although colonies arose on K$_R$ after overnight incubation and appeared on plates containing antibiotic after 2 days, plates were not scored for transformation frequency until after approximately 5 days of growth.

Generation of nonoxidizing transposon mutants of SG-1. The Tn917 portion of pLTV1 carries erythromycin resistance (Em^r), while the replicon portion bears tetracycline resistance (Tc^r). pLTV1 was transformed into SG-1, selecting for Em^r, and several of the transformants were stored in frozen culture with 10% (vol/vol) glycerol. An effort was made to minimize the chances of a single early insertional event dominating cultures by inoculating high-temperature cultures with several separate 30°C cultures. Just prior to a transposon mutagenesis experiment, one of the transformants was raised from frozen stock on R agar containing tetracycline (60 μg/ml) and erythromycin (0.15 μg/ml). After overnight growth at 30°C, a single colony each was used to inoculate four 5-ml cultures grown at 30°C in R medium containing tetracycline (60 μg/ml) and erythromycin (0.15 μg/ml) to an optical density at 660 nm of approximately 0.4. Each of these was used to inoculate (1/800) roughly a quarter each of 95 different 45°C cultures in R medium containing only erythromycin (0.15 μg/ml). These cultures were grown to stationary phase and then were plated onto K (sporulation) medium containing erythromycin (0.15 μg/ml) to screen for nonoxidizing mutants. Nonoxidizing mutants were identified as those colonies which did not turn brown after sporulation. Each of the 95 independent 45°C cultures was termed a transposon library. Em^r colonies were patched onto tetracycline-containing plates to screen for loss of pLTV1 from integrants. Transposition frequency was determined as follows in a manner similar to that previously reported (8). A 30°C mid-log-phase culture (grown as described above) was plated directly on K medium containing erythromycin (0.15 μg/ml) at 45 and at 30°C. The transposition frequency was calculated as the titer of Em^r, Tc^s colony-forming units at 45°C divided by the titer of Em^r colonies at 30°C.

Southern hybridization analysis of transposon mutants. Chromosomal DNA from SG-1 and its mutants was isolated either by a standard method for *B. subtilis* (11), which used phenol-chloroform extractions to purify DNA, or by the lysis method described by Young and Wilson (49) (omitting sodium dodecyl sulfate [SDS]) followed by purification of the DNA with CsCl-gradient centrifugation.

The areas surrounding the transposon inserts were restriction mapped by probing digests of chromosomal DNA with oligonucleotides made to the ends of Tn917-LTV1: *lacZ* end, Tn917(186), 5'-GATGTCACCGTCAAGTTAAATGTAC-3'; Tn917 end, Tn917(5192), 5'-GGGAGCATATCACTTTTCT TGGAG-3'. The location of these probes on Tn917-LTV1 in relation to the restriction enzymes used is shown in Fig. 1. The oligonucleotides were end labeled with T4 polynucleotide kinase (U.S. Biochemicals) and [γ-^{32}P]ATP, and Southern hybridizations were performed at 60°C either with dried gels or with gels that had been transferred to nylon membranes (Hybond-N; Amersham Corporation) by standard protocols (39). The nonoxidizing mutant SG1LTM2, as it was originally isolated after initial transposon mutagenesis, although Tc^s, was found in early Southern hybridization experiments to be carrying a truncated form of pLTV1 and was therefore cured of this plasmid by two rounds of growth at 43°C on R medium without antibiotics before being analyzed.

Isolation of regions surrounding transposon inserts. Taking advantage of the ColE1 replicon on Tn917-LTV1 (Fig. 1), the regions flanking the *lacZ* proximal end of transposon inserts from five of the nonoxidizing mutants were cloned as described
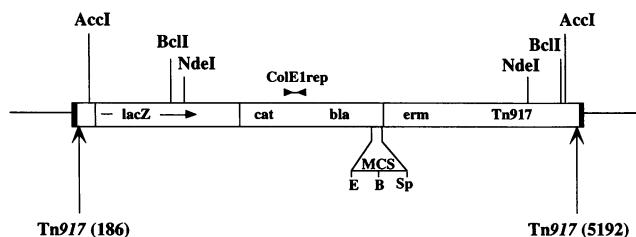
FIG. 1. Diagram representative of a Tn917-LTV1 (8) chromosomal insertion. The locations of probes used in Southern hybridization analysis are shown with respect to the restriction enzymes used (AccI, BclI, and NdeI). Also shown are the ColE1 replicon and multiple cloning site (MCS) used to clone the left flanking region from many of the transposon mutants. Only the AccI, BclI, and NdeI sites closest to the ends of the transposon are indicated; other sites may be present. Abbreviations: cat, chloramphenicol acetyltransferase gene; bla, β-lactamase gene; erm, Tn917 ribosomal methyltransferase gene; E, EcoRI; B, BamHI; Sp, SphI.

by Camilli et al. (8). Genomic DNA from SG1LTM7, 14, 17, 24, and 26 was digested with EcoRI (which cuts once within the multiple cloning site adjacent to the ColE1 replicon), and the products were self-ligated (T4 DNA ligase; Bethesda Research Laboratories) in 120-μl ligation mixtures at a DNA concentration of 8.3 μg/ml. The ligations were ethanol precipitated and resuspended in water. The entirety of each ligation was electroporated into E. coli HB101 (Invitrogen Corporation Porator device, with E. coli prepared and electroporated as recommended by the manufacturer), and transformants were selected by their resistance to ampicillin (Ap^r [encoded by the bla gene on Tn917-LTV1]). The resulting plasmids were designated pLVM7E, 14E, 17E, 24E, and 26E. To isolate the NdeI fragments carrying these genes without transposon inserts, the small EcoRI-SalI fragment from two of the resulting plasmids (pLVM17E and 26E), the portion of the plasmids carrying chromosomal loci, was isolated from a low-melting-point agarose gel and labeled with [α-³²P]dCTP by random priming (Random Primed DNA Labeling Kit; Boehringer Mannheim Biochemicals). These fragments were then used to probe colonies of E. coli HB101 carrying an NdeI library of wild-type SG-1 genomic DNA in pBR322 (NdeI fragments of total genomic SG-1 DNA cloned into the unique NdeI site of pBR322) by standard protocols (39). The constructs recovered by probing with pLVM17E and pLVM26E were designated pBRM17N and pBRM26N, respectively.

Complementation of a nonoxidizing mutant. The plasmid pGK13 (28) was used to deliver wild-type genes into one of the nonoxidizing mutants for complementation analysis. pGK13 has been shown to replicate in a wide variety of hosts, both gram negative and gram positive, and therefore E. coli could be used as an intermediate in constructing recombinant pGK13 plasmids. This was necessary because of the low transformation efficiency of SG-1 and the need to use large amounts of plasmid DNA to transform SG-1. pBRM17N was digested with NdeI and blunt ended with the Klenow fragment of E. coli DNA polymerase (39), and the 8.6-kb fragment was gel purified from low-melting-point agarose with GELase (Epicentre Technologies). Because pGK13 does not carry a convenient NdeI or blunt-ended site, the region was first cloned into the super-linker region of pSE380 (Invitrogen Corporation) and then was cleaved with restriction enzymes with sites on either side of the insert that are compatible to the desired cloning site in pGK13. The blunt-ended 8.6-kb fragment was ligated with StuI-digested, phosphatase-treated (shrimp alkaline phos-

phatase; U.S. Biochemical Corporation) pSE380. The ligation mixture was transformed into HB101 made competent for DNA uptake by calcium chloride treatment (39), selecting for Ap^r. The resulting plasmid was isolated, digested with XbaI and SpeI, and ligated with NheI-digested, phosphatase-treated pGK13. The recombinant pGK13 plasmid was transformed into E. coli (Epicurian coli Supercompetent XL1-Blue; Stratagene) and selected with erythromycin at 100 μg/ml. The resulting plasmid DNA was then isolated and transformed into SG1LTM22, selected with chloramphenicol, and screened for its ability to restore manganese oxidation.

RESULTS

Protoplast transformation of SG-1. Trials involving the development of a transformation system in SG-1 were performed with the plasmid pIL253 (42). pIL253 is a high-copy-number derivative of the streptococcal plasmid pAMβ1 that has been shown to be stably maintained in a number of gram-positive hosts (26). Because SG-1 did not appear to be naturally competent for DNA uptake and several attempts to electroporate SG-1 with plasmid DNA were unsuccessful, protoplast transformation was employed as a method to mobilize plasmids into SG-1. Protoplasting involves the digestion of the cell wall in an isotonic medium. Because SG-1 is a marine bacterium that will not grow without seawater salts in the medium, the buffers and regeneration medium used during protoplast transformation of B. subtilis (9) were modified to mimic the components of the seawater-based K medium usually used to culture SG-1. The initial transformation was performed essentially as outlined in Materials and Methods, except that lysozyme was used at a concentration of 2 mg/ml and PEG with a molecular weight of 6,000 was used. Transformants were selected by their Em^r (encoded by pIL253), and transformation was confirmed by purification of the plasmid from a random selection of transformants. Analysis of isolates showed that each carried an intact copy of the plasmid with the expected restriction enzyme fragment patterns (Fig. 2). A transformation efficiency of 44 transformants per μg of DNA was obtained with only 0.02% of the number of cells in the original culture appearing as colonies on regeneration plates without antibiotic (percentage of regeneration). Further experiments established that both digestion with lysozyme and the presence of PEG were required for efficient transformation of SG-1 (Table 2).

Numerous attempts to optimize the transformation procedure met with little success. In initial experiments, it was assumed that because SG-1 is a marine bacterium, it would be necessary to include seawater salts in media used during protoplasting. To test this assumption, the original protoplasting buffer was altered to match more closely that which is used for B. subtilis, a 0.5 M sucrose–20 mM MgCl₂ buffer containing 2× Penassay broth (9). When a 0.5 M sucrose–20 mM MgCl₂ buffer containing 2× K medium nutrients without seawater was used as the protoplasting buffer (SMA− [see Materials and Methods]), the survival and transformation efficiency dropped severalfold compared to the values obtained when the seawater-based buffer (1/2S)MAK was used. The results were a very low level of regeneration (0.00005%) and essentially no transformation (three transformants per μg of DNA). Isolation of pIL253 from SG-1 and reintroduction gave no higher level of transformation than that isolated from its original host, B. subtilis 642. A relatively small increase in transformation efficiency (from 44 to 221 transformants per μg of DNA) could be achieved when the lysozyme concentration during protoplasting was lowered from 2 mg/ml to 0.5 mg/ml. Whether PEG
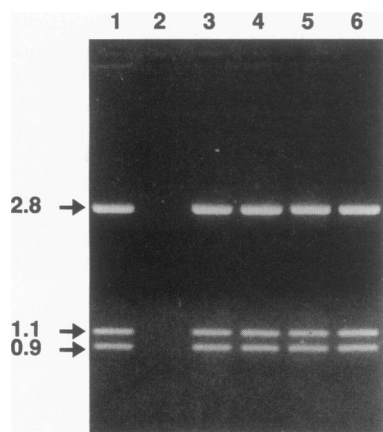
FIG. 2. *Hha*I restriction fragment pattern of pIL253 isolated from SG-1 transformants after electrophoresis through a 0.8% agarose gel. Lanes: 1, pIL253 from *B. subtilis* 642; 2, plasmid preparation from wild-type SG-1; 3 to 6, plasmid DNA from four randomly selected Em^r isolates of SG-1 after transformation with pIL253. The sizes (in kilobases) of the fragments are indicated (arrows).

was used at a molecular weight of 1,000, 6,000, or 8,000 made no difference in the transformation efficiency. If the expression period after transformation was omitted, then a slightly lower level of regeneration was seen and no transformation was achieved. When a culture containing 1.2×10^{10} cells per ml (100%) was protoplasted in (1/2S)MAK, the number of protoplasts seen under the light microscope was 5.4×10^9/ml (45%), while after plating on K_R medium the number of total colonies was 2.3×10^7/ml (0.3%). This suggests that the low level of survival and the correspondingly low level of transformation were not primarily due to problems in stability of the protoplasts during their formation but rather were due to problems associated with regeneration.

Nevertheless, by the method given in Materials and Methods, a modest level of transformation was routinely achieved, with usual transformation efficiencies ranging from 30 to 430 transformants per μg of DNA. The percentage of cells surviving the procedure was always very low, often with less than 0.1% of the original cells appearing as colonies on regeneration plates. No deletions or rearrangements have ever been seen in pIL253 (Fig. 2) or any of the other plasmids isolated from SG-1 as determined by agarose gel electrophoresis.

Transposon mutagenesis of SG-1. Because pLTV1 is temperature sensitive for replication, the plasmid is eliminated

TABLE 2. Conditions required for protoplast transformation of SG-1[a]

Treatment			Regeneration (%)[b]	Transformation efficiency (no. of transformants/μg of DNA)
Lysozyme	PEG	pIL253		
−	+	+	10	0
+	−	+	ND[c]	3.4
+	+	−	0.05	0.17
+	+	+	0.003–0.03	96–430

[a] Data are a compilation of results from several experiments in which protoplasting was carried out in (1/2S)MAK with or without 1 mg of lysozyme per ml and transformation was performed in the presence or absence of 40% PEG 1000 in 1× SMA.
[b] Calculated as the percentage of total CFU present in the sample relative to the number of cells present in the original culture.
[c] ND, not determined.

from mutagenic libraries by outgrowth at elevated temperatures. It was necessary to use a temperature of 45°C (the maximum growth temperature for SG-1) to block replication of pLTV1 in SG-1. Growth at temperatures lower than 45°C (42°C) resulted in less plasmid loss (~50% plasmid loss). We also found it necessary to use only low levels of erythromycin (the selection on Tn917) to obtain efficient plasmid loss. For example, if 1 μg of erythromycin per ml was used (instead of 0.15 μg/ml) during the high-temperature outgrowth and subsequent mutant isolation, then 100% of the isolates retained the Tc^r phenotype carried on the replicon portion of pLTV1, indicating the continued presence of the plasmid. Presumably the higher level of erythromycin was selecting for maintenance of the plasmid. On the other hand, if no antibiotic was used, then the plasmid was lost from the culture, but no transposition occurred. This is probably due to the fact that erythromycin must be present at some level to induce transposition of Tn917 (46). The loss of pLTV1 from SG-1 cultures at 45°C in 0.15 μg of erythromycin per ml was 98%, and the transposition frequency of Tn917-LTV1 in SG-1 averaged $\sim 1 \times 10^{-3}$.

From 95 transposon libraries, 27 nonoxidizing yet still sporulating mutants (usually termed nonoxidizing, or Mnx for Mn oxidation) were isolated, each from a different library. These were designated SG1LTM1 to SG1LTM27. The nonoxidizing mutants could easily be identified after sporulation as those which remained white rather than the characteristic brown of manganese oxide. Colonies which did not oxidize simply because they did not sporulate appeared transparent and were not chosen for study. Sporulation by the nonoxidizing mutants was confirmed by the detection of phase-bright endospores with light microscopy. Nonoxidizing mutants arose at a frequency of 0.35% of all transposon mutants.

Restriction mapping of transposon inserts. The locations of transposon insertions in the mutants were mapped by Southern blot analyses (Fig. 3 and Tables 3 and 4). Chromosomal DNA from transposon mutants was probed with oligonucleotides homologous to the ends of the transposon. The DNA was digested with enzymes with sites at both ends of Tn917-LTV1, creating hybridizing fragments that were linked to flanking regions of genomic DNA (Fig. 1). The restriction enzymes used were *Bcl*I, *Acc*I, and *Nde*I, which cleave the transposon 1.6, 0.3, and 3.2 kb from the *lacZ* end and 0.7, 0.2, and 2.2 kb from the Tn917 end, respectively. Such probing resulted in two hybridizing fragments (Fig. 3), indicating the presence of a single insertion of the transposon in each mutant. The sum of the sizes of the two fragments minus the portion attributable to the ends of the transposon gave the size of the restriction fragment in which the transposon resided (Table 3). Probing with only one of the oligonucleotides highlighted one of the flanking regions and thus allowed us to orient the transposon within each restriction fragment.

Using such analyses, we found that many of the 27 Mnx mutants under study had insertions within the same-size restriction fragments (Table 3). Seventeen had inserts which clustered within two loci; 13 were located in an 8.6-kb *Nde*I region within 3 kb of each other, and another 4 were located in a 5-kb *Nde*I region within 500 bp of each other (designated Mnx regions; Fig. 4). The remaining 10 mutants did not map to these regions, nor did they map close to each other. It is possible that transposon insertions were clustered in these regions because they were hot spots for transposon insertion, had nothing to do with manganese oxidation, and the loss of oxidation was caused by spontaneous mutation. However, it is unlikely that all of the nonoxidizing mutants were the result of spontaneous mutations, given the high frequency of nonoxidizing mutants obtained during transposon mutagenesis. To rule
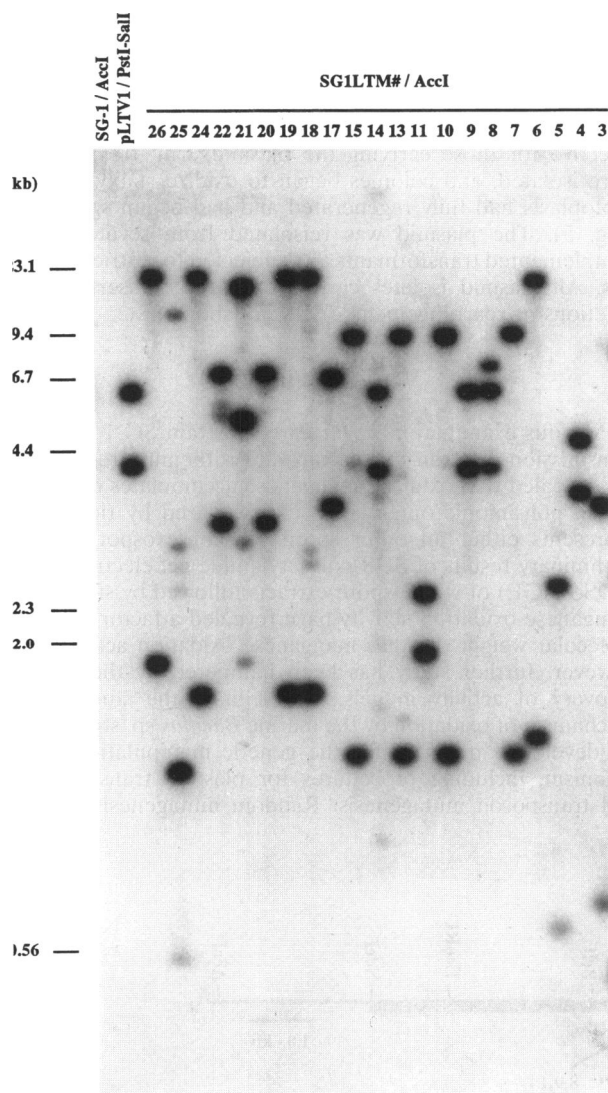
FIG. 3. Representative autoradiogram from Southern hybridization analyses of transposon mutants. DNA was digested, subjected to electrophoresis through a 0.8% agarose gel, and probed with ^{32}P-labeled oligonucleotides homologous to sequences at each end of the transposon. Chromosomal DNA from SG-1 and nonoxidizing mutants was digested with *Acc*I that cuts the transposon internally (relative to the oligonucleotides) at both ends (Fig. 1). pLTV1, cleaved with *Pst*I and *Sal*I (such that each end of the transposon on the plasmid is on a different fragment), is shown as a positive control. Size markers (in kilobases) are given for reference. The lowest bands (~0.56 kb) in SG1LTM5 and 25 did not hybridize well but represent positive signals. The highest band in SG1LTM8 is the result of a partial digest.

out the possibility that the clustered regions represented a hot spot phenomenon, 18 mutants which still oxidize manganese were isolated from the same libraries as the nonoxidizing mutants and were subjected to restriction analysis as described above. None of these oxidizing mutants mapped to the same areas as any of the Mnx mutants and, in fact, within these mutants the transposon seemed to have inserted randomly (Table 4).

All of the integrants analyzed above (both oxidizing and nonoxidizing) were tested for β-galactosidase activity by plating on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Wild-

TABLE 3. Sizes of restriction fragments disrupted by transposon insertion in manganese oxidation mutants[a]

Strain	Fragment size (kb) of[b]:		
	*Nde*I	*Acc*I	*Bcl*I
SG1LTM7	8.5	9.9	3.2
SG1LTM10	8.5	10.6	3.2
SG1LTM13	8.3	10.3	3.2
SG1LTM15	8.5	9.8	3.2
SG1LTM16	8.1	10.1	2.9
SG1LTM17	8.3	9.9	1.7
SG1LTM20	8.6	9.7	1.5
SG1LTM22	8.6	9.8	1.5
SG1LTM23	8.0	9.6	1.5
SG1LTM2	9.1	10.3	1.6
SG1LTM8	8.5	10.0	1.1
SG1LTM9	8.5	10.0	1.0
SG1LTM14	8.4	9.8	1.1
SG1LTM18	5.1	≥17.6[c]	0.42
SG1LTM19	5.1	≥18.1	0.39
SG1LTM24	5.1	≥17.6	0.39
SG1LTM26	5.1	≥17.6	0.37
SG1LTM4	11.8	8.0	1.6
SG1LTM3	7.9	3.8	2.1
SG1LTM11	7.1	4.2	5.5
SG1LTM1	6.6	16.8	2.7
SG1LTM5	6.3	2.8	0.51
SG1LTM12	4.6	5.4	2.8
SG1LTM25	4.5	1.4	1.9
SG1LTM21	4.1	20.9	9.1
SG1LTM6	1.7	15.2	16.1
SG1LTM27	1.3	8.2	3.9

[a] Data are the result of Southern hybridization analyses of chromosomal DNA probed with Tn917 as described in the text.
[b] Similarly sized fragments are boxed.
[c] In samples with a "≥" symbol, the larger of the two hybridizing fragments in each case was located at the top of the digest, therefore it was impossible to determine an absolute fragment size.

type SG-1 had no detectable level of β-galactosidase activity. Five of the 18 oxidizing mutants showed *lacZ* activity. Of the 27 nonoxidizing mutants, 5 were positive. All of the mutants with insertions in the Mnx regions were *lacZ* negative, except SG1LTM2, which was *lacZ* positive and which was oriented in the opposite direction (Fig. 4).

Complementation of a nonoxidizing mutant. To help confirm that the Mnx regions are indeed involved in manganese oxidation, we complemented the oxidizing defect in one of these mutants (Fig. 5). The 8.6-kb *Nde*I region was chosen for this purpose because it was a large fragment, extending for some distance on both sides of the cluster of transposon inserts, and was therefore likely to contain any control elements for the region. Because pIL253 confers Em[r] and the transposon mutants were selected on the basis of their Em[r], pIL253 was not used for cloning into the transposon mutants. Transformation of SG-1 with several possible cloning vectors bearing Cm[r] markers was attempted. These included pBS19 (19), pMTL500C (43), pHV1431 (26), and pHPS9 (21). However, with all of these plasmids, while antibiotic-resistant colonies appeared on regeneration plates, for an unknown reason putative transformants would not maintain their antibiotic resistance upon transfer. It was found, though, that the lactococcal plasmid pGK13 (28) could be transferred to SG-1, selecting for Cm[r]. A very low transformation efficiency (around 10 transformants per μg of DNA) was obtained, but the plasmid appeared to be stably maintained. The recombinant pGK13 plasmid carrying the 8.6-kb *Nde*I Mnx fragment

TABLE 4. Sizes of restriction fragments disrupted by transposon insertion in mutants which oxidize manganese[a]

Strain[b]	Fragment size (kb) of:		
	NdeI	AccI	BclI
86-6	16.5	2.4	10.0
86-3	11.9	2.5	1.8
70-8	11.8	3.0	ND[c]
70-4	10.0	1.7	10.2
86-7	6.4	1.3	8.6
58-2	5.3	4.9	2.4
58-1	5.1	7.6	1.7
70-2	5.0	1.4	5.0
58-4	4.9	3.0	2.7
70-7	4.6	3.6	0.44
58-6	4.5	7.6	1.8
58-8	4.3	5.5	4.3
70-3	4.3	5.5	2.7
58-5	4.0	1.5	1.1
58-7	3.7	4.7	5.2
70-5	3.6	4.0	2.1
86-5	3.6	23.1	13.6
70-6	ND	ND	3.6

[a] Data are the result of Southern hybridization analyses of chromosomal DNA probed with Tn917 as described in the text.

[b] Oxidizing mutants beginning with 58 came from the same transposon library as SG1LTM8, those beginning with 70 came from the same transposon library as SG1LTM15, and those beginning with 86 came from the same transposon library as SG1LTM22.

[c] ND, not determined.

(pGKM17N) was constructed in *E. coli*, isolated, and transformed into the nonoxidizing mutant SG1LTM22, selecting for Cm^r. Tn917-LTV1 also carries a Cm^r marker, making transposon mutants resistant to 3 μg of chloramphenicol per ml, but we found that a higher level of chloramphenicol (10 μg/ml) was selective for those carrying the plasmid. Cm^r transformants were isolated, and colonies began to oxidize stably once the protoplasts had fully regenerated and had begun sporulating (Fig. 5). The plasmid was reisolated from several of the complemented transformants and subjected to restriction analysis. All plasmid isolates carried the proper insert with no deletions or rearrangements (data not shown).

DISCUSSION

Previous examination of *Bacillus* sp. strain SG-1 spores by transmission electron microscopy and ruthenium red staining has revealed that oxidized manganese accumulates on a laminated, polyanionic outer layer, characterized by ridges, that represents either an outer spore coat or exosporium (44). Preliminary results of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of whole-spore extracts followed by staining for manganese oxidation activity have revealed a factor with high molecular weight that has manganese oxidation activity (45); however, further study has been hampered by the variable recovery of activity in gels. To facilitate the study of the mechanism of oxidation by the marine *Bacillus* sp. strain SG-1, we developed methods for the genetic manipulation of the organism, including procedures for plasmid transformation and transposon mutagenesis. Random mutagenesis of SG-1
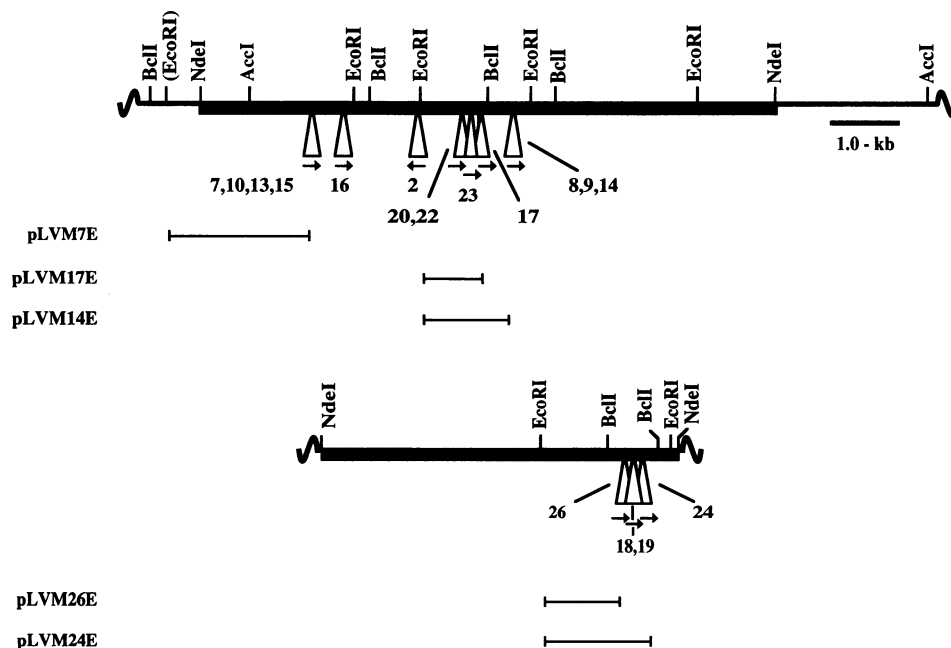

FIG. 4. Restriction maps of SG-1 chromosomal regions showing where many of the Tn917 insertions corresponding with a non-manganese-oxidizing phenotype clustered (Mnx regions). Sites of Tn917 insertions are indicated by the open triangles along with the number assigned to the respective mutant in each case. The transcriptional orientation of the transposon-encoded *lacZ* gene is designated by the arrow beneath each transposon insertion. Fragments that have been cloned from these regions with the ColE1 replicon in the transposon are shown below the region along with the name given the newly generated plasmid in each case. pLVM17E and pLVM26E were used as probes to isolate the 8.6- and the 5-kb *Nde*I regions, respectively, from wild-type SG-1 (indicated by the black bars). The 8.6-kb *Nde*I region was then used to complement SG1LTM22. The *Eco*RI site outside the 8.6-kb *Nde*I fragment is in parentheses because other *Eco*RI sites may exist outside the cloned regions. Only the restriction sites relevant to this report are shown.
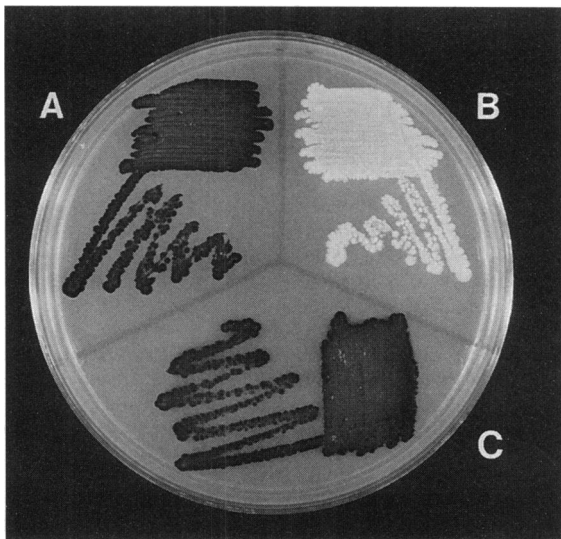
FIG. 5. Complementation of a manganese oxidation mutant of SG-1. Colonies are shown after sporulation at 30°C on K medium supplemented with chloramphenicol. (A) Wild-type SG-1 (carrying pGK13). (B) One of the nonoxidizing transposon mutants, SG1LTM22 (pGK13). (C) The complemented mutant, SG1LTM22(pGKM17N).

with Tn917 allowed generation of nonoxidizing (Mnx) mutants, mapping of which revealed two regions of the chromosome (~3.5 kb) that are involved in the oxidation of manganese.

Protoplast transformation has proven to be a useful method of gene transfer in gram-positive bacteria (24). Transformation efficiencies vary greatly depending on the species, with reported values for Bacillus spp. ranging from, for example, 5 × 10^2 transformants per μg of DNA in Bacillus licheniformis and Bacillus amyloliquefaciens (20) to 4 × 10^7 transformants per μg of DNA in B. subtilis (9). The protoplast transformation procedure described here allowed transformation of SG-1 at an efficiency of around 100 transformants per μg of plasmid DNA. As has been noted for protoplast transformation of other species, digestion of the cell wall with lysozyme, the presence of PEG, and an incubation period prior to plating to allow expression of antibiotic resistance were all required for successful transformation. We tested to see whether perhaps some active restriction-modification barrier was preventing efficient transformation of SG-1. Transformation of pIL253 derived from SG-1 gave the same efficiency of transformation as that isolated from B. subtilis, suggesting that the plasmid had not been modified in SG-1 and restriction was not responsible for the low transformation efficiency. We have not tried curing previously transformed SG-1 of its plasmid and retransforming that strain to see whether it might have obtained some defect in a restriction system. Because the major bottleneck in optimization of the procedure appears to lie in the regeneration, future experiments to optimize transformation efficiencies may concentrate on improving regeneration, perhaps by including osmoprotectants or cell wall material in the medium.

To our knowledge, this is the first report in which transformation of a marine gram-positive bacterium has been described. To obtain a significant level of transformation and regeneration of SG-1 protoplasts required the use of a seawater buffer. Therefore, although not tested, this technique might be useful in the transformation of other marine gram-positive bacteria, many of which are known to produce useful natural products (2).

We succeeded in generating transposon mutants of SG-1 with Tn917-LTV1, each of the mutations being a result of a single transposition event. To be a useful tool for mutagenesis, a transposon must insert randomly throughout the genome. A commonly used method for determining the randomness of insertion of a transposon in any particular organism is to measure the frequency at which various auxotrophic mutations can be isolated. This method could not be applied to SG-1 because a defined minimal medium for growth of this organism has not yet been developed. However, on the basis of the results of probing the transposon mutants which still oxidize manganese, the transposon appeared to be inserting randomly in the chromosome.

A fairly high frequency of nonoxidizing mutations (0.35%) was observed during mutagenesis experiments. This could be explained in one of two ways. Either a relatively large amount of DNA is devoted to manganese oxidation in SG-1, or the regions involved in the oxidation contain sequences that are hot spots for transposon insertion. A result similar to the latter was seen during initial testing of Tn917-LTV3 (a construct similar to Tn917-LTV1) in Listeria monocytogenes (8). Camilli et al. found that although Tn917-LTV3 inserts fairly randomly throughout the chromosome, it inserts into some sites, including the hemolysin gene (hlyA), more frequently than into others.

The majority of Tn917 insertions generating a nonoxidizing phenotype in SG-1 clustered within two regions of the chromosome. None of the insertions in mutants that still oxidize manganese mapped to these regions. This strongly suggested that these areas are somehow involved in manganese oxidation. Further evidence for this was supplied by complementation of one of the nonoxidizing mutants from one of the regions. Within the Mnx regions, there are several instances in which different mutants carry transposon insertions in identical sites despite the fact that the mutants were isolated from different transposon libraries. This suggests that there are local hot spots for insertion of the transposon within these regions. Transposons have been seen in some instances to have specificity for certain sequences. For instance, Tn3, the family to which Tn917 belongs (37), has been shown to have a tendency to insert into A-T-rich regions of the chromosome (41).

Southern hybridizations with probes specific to either end of the transposon not only allowed the size of the restriction fragment into which the transposon had inserted to be determined but also allowed the determination of the position and orientation of the transposon within each restriction fragment. For an unknown reason, the majority of inserts within a given Mnx region were oriented in the same direction (Fig. 4). This suggests that, in these regions, there may have been some selection for or against a particular orientation or that some specificity on the part of the transposase tended to cause the transposon in these regions to insert in a certain direction. Perhaps this phenomenon is related to the direction of transcription within these regions. This sort of orientational specificity has been seen for Tn5 insertions in actively transcribed regions (3). However, Camilli et al. (8), using the Tn917-LTV3 construct, found that within the hlyA gene, a strongly expressed gene, four of the eight inserts were oriented in one direction and four were oriented in the other. Of the mutants in the two Mnx regions, all except SG1LTM2 were lacZ negative. Therefore, it seems that within the Mnx regions, in most cases, the transposon-encoded lacZ gene was oriented in the wrong direction to create transcriptional fusions to these genes.

Although we have demonstrated that the Mnx loci are somehow involved in manganese oxidation, it is possible that these genes do not encode the oxidizing factors themselves but

perhaps encode something indirectly related to the process. The nonoxidizing mutants form phase-bright spores with no obvious phenotypic differences from wild-type spores other than their inability to oxidize manganese. It is therefore likely that these loci encode factors that are closely linked to the oxidation process. Analysis of the mutants is currently being carried out to identify what these regions encode and what role they play in the oxidation of manganese by SG-1. Sequence analysis of these regions will reveal the number of genes involved, their organization, and whether they seem to encode primary products of hydrophobic nature as one would expect from spore coat or exosporium proteins (30, 48).

ACKNOWLEDGMENTS

REFERENCES

1. **Aoki, H., and R. A. Slepecky.** 1973. Inducement of a heat-shock requirement for germination and production of increased heat resistance in *Bacillus fastidiosus* spores by manganous ions. J. Bacteriol. **114:**137–143.
2. **Austin, B.** 1989. Novel pharmaceutical compounds from marine bacteria. J. Appl. Bacteriol. **67:**461–470.
3. **Berg, D. E., and C. M. Berg.** 1983. The prokaryotic transposable element Tn5. Bio/Technology **1:**417–435.
4. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
5. **Boyer, H. W., and D. Roulland-Dassiox.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli.* J. Mol. Biol. **41:**459–472.
6. **Brosius, J.** 1989. Superlinkers in cloning and expression vectors. DNA **8:**759–777.
7. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
8. **Camilli, A., D. A. Portnoy, and P. Youngman.** 1990. Insertional mutagenesis of *Listeria monocytogenes* with a novel Tn917 derivative that allows direct cloning of DNA flanking transposon insertions. J. Bacteriol. **172:**3738–3744.
9. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168:**111–115.
10. **Charney, J., W. P. Fisher, and C. P. Hegarty.** 1951. Manganese as an essential element for sporulation in the genus *Bacillus.* J. Bacteriol. **62:**145–148.
11. **Cutting, S. M., and P. B. V. Horn.** 1990. Genetic analysis, p. 27–74. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biology methods for *Bacillus.* John Wiley & Sons, Chichester, United Kingdom.
12. **de Vrind, J. P. M., F. C. Boogerd, and E. W. de Vrind-de Jong.** 1986. Manganese reduction by a marine *Bacillus* species. J. Bacteriol. **167:**30–34.
13. **de Vrind, J. P. M., E. W. de Vrind-de Jong, J.-W. H. de Voogt, P. Westbroek, F. C. Boogerd, and R. A. Rosson.** 1986. Manganese oxidation by spores and spore coats of a marine *Bacillus* species. Appl. Environ. Microbiol. **52:**1096–1100.
14. **Donovan, W., L. Zheng, K. Sandman, and R. Losick.** 1987. Genes encoding spore coat polypeptides from *Bacillus subtilis.* J. Mol. Biol. **196:**1–10.
15. **Ehrlich, H. L.** 1990. Geomicrobiology of manganese, p. 347–440. *In* H. L. Ehrlich (ed.), Geomicrobiology. Marcel Dekker, New York.
16. **Eisenstadt, E., S. Fisher, C.-L. Der, and S. Silver.** 1973. Manganese transport in *Bacillus subtilis* W23 during growth and sporulation. J. Bacteriol. **113:**1363–1372.
17. **Emerson, S., S. Kalhorn, L. Jacobs, B. M. Tebo, K. H. Nealson, and R. A. Rosson.** 1982. Environmental oxidation rate of manganese(II): bacterial catalysis. Geochim. Cosmochim. Acta **46:**1073–1079.
18. **Ghiorse, W. C.** 1984. Biology of iron- and manganese-depositing bacteria. Annu. Rev. Microbiol. **38:**515–550.
19. **Grey, G.** Unpublished observations.
20. **Grosch, J. C., and K. L. Wollweber.** 1982. Transformation of *Bacillus lichiformis* and *Bacillus amyloliquefaciens* protoplasts by plasmid DNA, p. 97–105. *In* U. N. Streips, S. H. Goodgal, W. R. Guild, and G. A. Wilson (ed.), Genetic exchange: a celebration and a new generation. Marcel Dekker, New York.
21. **Haima, P., D. van Sinderen, H. Scholting, S. Bron, and G. Venema.** 1990. Development of a β-galactosidase α-complementation system for molecular cloning in *Bacillus subtilis.* Gene **93:**41–47.
22. **Hastings, D., and S. Emerson.** 1986. Oxidation of manganese by spores of a marine bacillus: kinetic and thermodynamic considerations. Geochim. Cosmochim. Acta **50:**1819–1824.
23. **Hodges, N. A., and M. R. W. Brown.** 1975. Properties of *Bacillus megaterium* spores formed under conditions of nutrient limitation, p. 550–555. *In* P. Gerhardt, R. N. Costilow, and H. L. Sadoff (ed.), Spores VI. American Society for Microbiology, Washington, D.C.
24. **Hopwood, D. A.** 1981. Genetic studies with bacterial protoplasts. Annu. Rev. Microbiol. **35:**237–272.
25. **Iichinska, E.** 1960. Some physiological features of asporogenous mutants of bacilli. Microbiology **29:**147–150.
26. **Janniere, L., C. Bruand, and S. D. Ehrlich.** 1990. Structurally stable *Bacillus subtilis* cloning vectors. Gene **87:**53–61.
27. **Kihm, D. J., M. T. Hutton, J. H. Hanlin, and E. A. Johnson.** 1990. Influence of transition metals added during sporulation on heat resistance of *Clostridium botulinum* 113B spores. Appl. Environ. Microbiol. **56:**681–685.
28. **Kok, J.** 1991. Special-purpose vectors for lactococci, p. 97–102. *In* G. M. Dunny, P. P. Cleary, and L. L. McKay (ed.), Genetics and molecular biology of streptococci, lactococci, and enterococci. American Society for Microbiology, Washington, D.C.
29. **Kok, J., J. M. B. M. van der Vossen, and G. Venema.** 1984. Construction of plasmid cloning vectors for lactic streptococci which also replicate in *Bacillus subtilis* and *Escherichia coli.* Appl. Environ. Microbiol. **48:**726–731.
30. **Koshikawa, T., M. Yamazaki, M. Yoshimi, S. Ogawa, A. Yamada, K. Watabe, and M. Torii.** 1989. Surface hydrophobicity of spores of *Bacillus* spp. J. Gen. Microbiol. **135:**2717–2722.
31. **Krumbein, W. E., and H. J. Altmann.** 1973. A new method for the detection and enumeration of manganese oxidizing and reducing microorganisms. Helgol. Wiss. Meeresunters. **25:**347–356.
32. **Marquis, R. E.** 1989. Minerals and bacterial spores, p. 247–274. *In* T. J. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley and Sons, New York.
33. **Marshall, K. C.** 1979. Biogeochemistry of manganese minerals, p. 253–292. *In* P. A. Trudinger and D. J. Swaine (ed.), Biogeochemical cycling of mineral-forming elements. Elsevier Scientific Publishing Co., New York.
34. **Nealson, K. H., and J. Ford.** 1980. Surface enhancement of bacterial manganese oxidation: implications for aquatic environments. Geomicrobiol. J. **2:**21–37.
35. **Nealson, K. H., R. A. Rosson, and C. R. Myers.** 1989. Mechanisms of oxidation and reduction of manganese, p. 383–411. *In* T. J. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley & Sons, New York.
36. **Nealson, K. H., B. M. Tebo, and R. A. Rosson.** 1988. Occurrence and mechanisms of microbial oxidation of manganese. Adv. Appl. Microbiol. **33:**279–318.
37. **Perkins, J. B., and P. J. Youngman.** 1984. A physical and functional analysis of Tn917, a *Streptococcus* transposon in the Tn3 family that functions in *Bacillus.* Plasmid **12:**119–138.
38. **Rosson, R. A., and K. H. Nealson.** 1982. Manganese binding and oxidation by spores of a marine bacillus. J. Bacteriol. **151:**1027–1034.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular

cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

40. **Schaeffer, P., and H. Ionesco.** 1960. Contribution a l'etude genetique de la sporogenese bacterienne. C. R. Acad. Sci. **251:**3125–3127.

41. **Sherratt, D.** 1989. Tn*3* and related transposable elements: site-specific recombination and transposition, p. 163–184. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.

42. **Simon, D., and A. Chopin.** 1988. Construction of a vector plasmid family and its use for molecular cloning in *Streptococcus lactis*. Biochimie **70:**559–566.

43. **Swinfield, T., J. D. Oultram, D. E. Thompson, J. K. Brehm, and N. P. Minton.** 1990. Physical characterization of the replication region of the *Streptococcus faecalis* plasmid pAMβ1. Gene **87:**79–90.

44. **Tebo, B. M.** 1983. Ph.D. thesis. University of California, San Diego.

45. **Tebo, B. M., K. Mandernack, and R. A. Rosson.** 1988. Manganese oxidation by a spore coat or exosporium protein from spores of a manganese(II) oxidizing marine bacillus, abstr. I-121, p. 201. Abstr. Annu. Meet. Am. Soc. Microbiol. 1988. American Society for Microbiology, Washington, D.C.

46. **Tomich, P. K., F. Y. An, and D. B. Clewell.** 1980. Properties of erythromycin-inducible transposon Tn*917* in *Streptococcus faecalis*. J. Bacteriol. **141:**1366–1374.

47. **van Veen, W. L.** 1973. Biological oxidation of manganese in soils. J. Microbiol. Serol. **39:**657–662.

48. **Wiencek, K. M., N. A. Klapes, and P. M. Foegeding.** 1990. Hydrophobicity of *Bacillus* and *Clostridium* spores. Appl. Environ. Microbiol. **56:**2600–2605.

49. **Young, F. E., and G. A. Wilson.** 1974. *Bacillus subtilis*, p. 69–114. *In* R. C. King (ed.), Handbook of genetics. Plenum Press, New York.